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14. ABSTRACT

TSPY is the putative gene for the gonadoblastoma locus on the Y chromosome (GBY). It is aberrantly expressed in and could contribute to prostate cancer. The objectives of the project are: 1) to identify the oncogenic and/or tumor promoting properties of TSPY, and 2) to correlate TSPY over-expression with prostatic oncogenesis in transgenic mice. During the funded period, we confirmed the involvement of TSPY in the initiation and/or early stages of prostate cancer and germ cell tumors. Ectopic TSPY expression potentiates cell proliferation (by shortening the G2/M phase) and tumor growth in nude mice, leading to genome instability and gene dysregulation. Numerous oncogenes were up regulated and growth inhibitors were repressed in these TSPY-expressing cells. TSPY binds to cyclin B and exerts activating effects on cyclin B-CDK1 kinase activities. TSPY interacts with the elongation factor 1A, thereby enhancing the protein synthetic machinery and exacerbating tumor cell growth. TSPY transgene was expressed in the hyperplasic region in the prostates of transgenic mice, thereby supporting its role(s) in either the initiation and/or early stages of prostatic carcinogenesis. Our results have provided significant insights on the role of TSPY in prostatic oncogenesis and development of diagnostic, therapeutic and preventive strategies for prostate cancer.

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INTRODUCTION

The TSPY gene is a tandemly repeated gene on the short arm of the human Y chromosome [1]. Genetic mapping and complete sequencing of the human Y chromosome demonstrated that TSPY is located in the critical region harboring the only oncogenic or tumor-promoting locus, termed gonadoblastoma on Y (GBY), on this male-specific chromosome [2-4]. GBY is postulated to serve a normal function(s) in the testis, but predisposes a dysgenetic gonad to gonadoblastoma development. Gonadoblastoma are benign germ cell tumors that develop most frequently in XY sex-reversed and intersex patients and men with testis dysgenesis [5, 6]. Gonadoblastoma, and the testicular carcinoma-in-situ (CIS) or intratubular germ cell neoplasia unclassified (ITGCNU) are considered to be premalignant precursors for germ cell tumors [7]. Mapping of TSPY to the critical region of GBY, therefore, presented TSPY as a candidate for this oncogenic locus. The TSPY tandem arrays constitute the most homogenously repeated coding sequences in the human genome and occupy from 0.4-1.0 MB of DNA on the human Y chromosome [8, 9]. They are also hot spots for genomic instability (with wide copy number variation among individuals) [9, 10] and epigenetic dysregulation (ectopically expressed in a variety of tumors, including various types of germ cell tumors and prostate cancer, and other somatic tumors, i.e. brain, skin and liver of male origin) [11-15]. TSPY serves vital functions in male stem germ cell renewal and meiotic division in the testis. When it is aberrantly expressed in tissues, such as the epithelial cells of the prostate, incapable of entering spermatogenesis, it exerts an oncogenic or tumor promoting effects, and in collaboration with other oncogenic events, leading to oncogenesis. The majority of our efforts in the project were focused on the two specific aims proposed in the original application.

BODY

TASK 1. TO IDENTIFY THE ONCOGENIC AND/OR TUMOR PROMOTING PROPERTIES OF TSPY

A. TSPY Expression in Tumor Tissues, including Prostate Cancer

a) Expression of TSPY in Gonadoblastoma (Reprint 6)

TSPY is a candidate gene for the GBY locus on the human Y chromosome. To establish its potential involvement in gonadoblastoma, we had examined its expression in several gonadoblastoma specimens form intersex, sex-reversed patients and men with dysfunctional testes, using immunostaining techniques. Our studies, collectively, demonstrate that TSPY is expressed in early and late stages of gonadoblastoma. It is co-expressed with numerous germ cell tumor markers, including OCT4, PLAP, and c-KIT and the proliferative marker, Ki-67, in the same tumor germ cells of these specimens. Significantly, TSPY could be used as a marker during the entire course of germ cell tumorigenesis, including early to late stages of gonadoblastoma development [16, 17]. These results demonstrate that TSPY could indeed be involved in the initiation (and predisposition) of germ cells to tumorigenesis.

b) TSPY is expressed in the premalignant precursor, CIS, and seminoma of testicular germ cell tumors (Reprints 6, 7)

As discussed before, gonadoblastoma and testicular CIS share significant similar properties and are precursors for malignant germ cell tumors. To explore the possibility that this GBY candidate gene is also involved in the germ cell tumorigenesis in testicular germ cell tumors, we

have examined its expression in various types of testicular germ cell tumors, including CIS, seminomas and nonseminomas (i.e. embryonal carcinoma, yolk sac tumors, and intracranial germ cell tumors. Our results demonstrate that TSPY is preferentially expressed in the precursor, CIS, seminomas and intracranial germ cell tumors of male origin, but not abundantly in nonseminomas (including embryonal carcinoma, yolk sac tumors and teratoma). These results are significant, suggesting that TSPY could participate in the early events of germ cell tumorigenesis, could exert its oncogenic effects seminoma (along the germ line phenotype), but could be less important when the germ cell tumors evolve into a more multipotent states of nonseminomas.

Using microarray expression analysis, we demonstrate that ectopic expression of TSPY up regulates genes located at the short arm of chromosome 12, which are frequently amplified in testicular germ cell tumors and have been postulated to be involved in germ cell tumorigenesis. These findings further suggest that TSPY could directly or indirectly participate in the regulation of genes important for germ cell tumorigenesis.

c) Expression of TSPY in Human Prostate Cancer (Reprint 1)

Previously, we have investigated the role of the Y chromosome in prostate cancer by examining the expression of 31 Y chromosome genes in prostate cancer of various degrees of malignancy and prostatic cell lines [13]. The results from this initial survey identified the TSPY gene to be a strong candidate gene from the male-specific chromosome that could potentially contribute to prostatic oncogenesis. TSPY was expressed at heterogeneous levels in prostatic samples of various degrees of malignancy. Its expression could be stimulated by androgen in the prostatic cell line, LNCaP.

We further studied the TSPY expression by in situ mRNA hybridization and immunostaining on prostatic sections from 11 patients suffering from prostate cancer of various Gleason grades [12]. Both RNA and protein studies corroborated with each others, demonstrate a

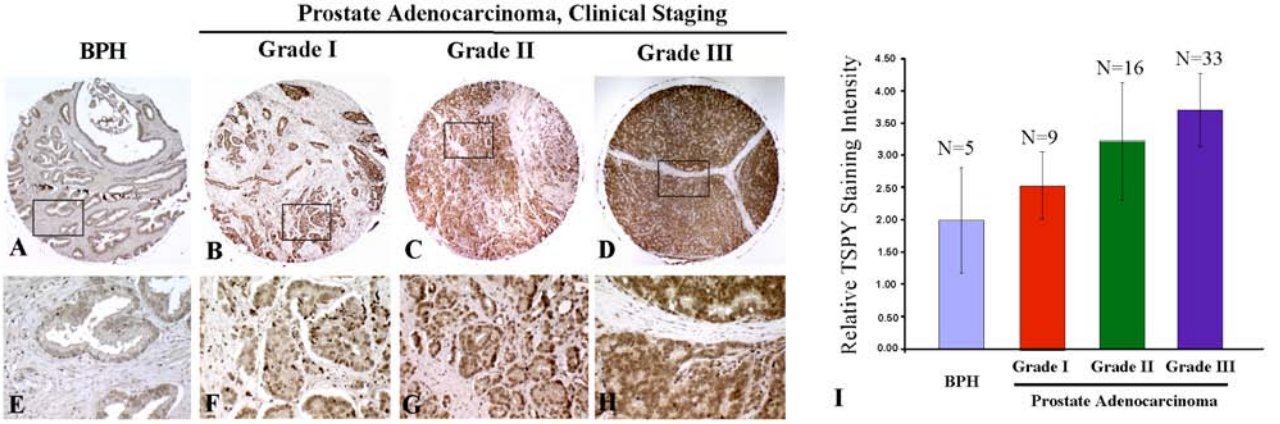


Figure 1. Immunostaining of BPH and various grades of prostate adenocarcinoma with TSPY antibody in a tissue array consisting of 63 samples. **A-D** show samples of BPH, grade I, II, and III prostate adenocarcinoma, boxed areas are represented in **E-H** respectively. **I** shows the relatively immunostaining intensity of TSPY in these samples. Grade I = well differentiated, Gleason score 6 or less; II = intermediate, Gleason ~ 7; III = high grade, Gleason > 8.

low-level expression of TSPY gene in morphologically normal epithelia, an elevated level in hyperplastic and cancer epithelia of low Gleason grades and the highest levels on cancer cells of high Gleason grades. Hence, there is a parallel increase in TSPY expression with malignancy in prostate cancer.

Recently, we had performed a similar immunostaining study on a tissue array consisting of 63 specimens of BPH and prostate cancer of various clinical stages (i.e. I, II and III). The intensity

of TSPY was scored on an arbitrary scale of 1 to 5, i.e. light to intense staining (Figure 1). Our results clearly confirm those of previous studies, establishing the fact that TSPY expression

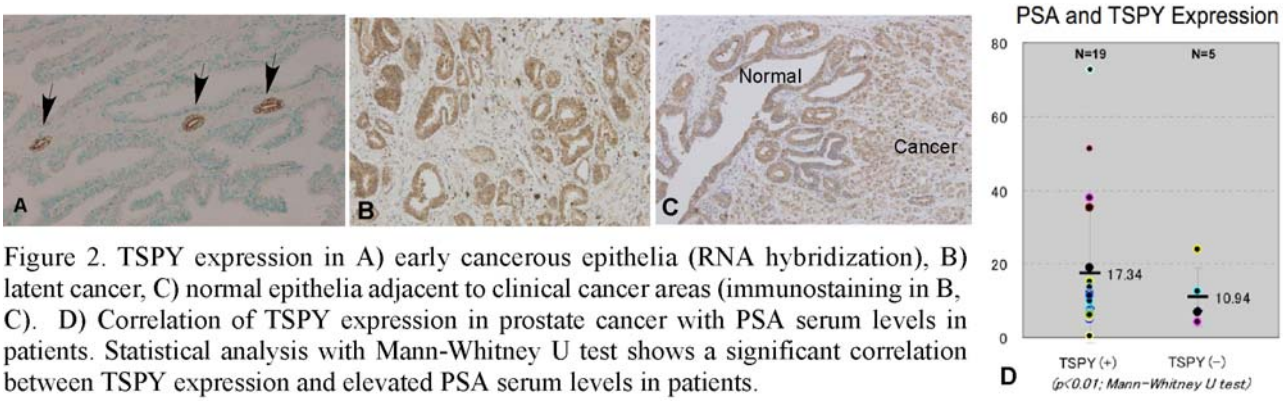


Figure 2. TSPY expression in A) early cancerous epithelia (RNA hybridization), B) latent cancer, C) normal epithelia adjacent to clinical cancer areas (immunostaining in B, C). D) Correlation of TSPY expression in prostate cancer with PSA serum levels in patients. Statistical analysis with Mann-Whitney U test shows a significant correlation between TSPY expression and elevated PSA serum levels in patients.

increases with the degree of malignancy among these prostate cancer samples.

In collaboration with Drs. Shingo Hatakeyama and Chikara Ohyama, Hirosaki University, we have conducted a more comprehensive study of >80 specimens, including various latent cancers from elderly patients with bladder, but not prostate, cancer. Our results demonstrate that TSPY expression is detected in early stages (Figure 2A) and increases with increasing clinical stages of prostate cancer, as observed before. Selected normal epithelia adjacent to cancer areas also showed positive TSPY expression (Figure 2C). Statistical analysis showed that there is a significant association of TSPY expression in clinical prostate cancer with elevated PSA serum levels in the corresponding patients (Figure 2D). Significantly, latent cancers in prostates of patients suffering from other diseases also showed TSPY expression at moderate to high levels (Figure 2B).

The results are important. They confirm our previous findings on TSPY expression in prostate cancer and further demonstrate TSPY expression could also be observed in phenotypically normal epithelia, adjacent to cancerous lesions, and in latent prostatic cancer foci of patients who do not show any symptoms of clinical prostate cancer. Collectively, these results are parallel with those gonadoblastoma, CIS and testicular germ cell tumors, i.e. TSPY could be expressed in premalignant forms of prostate cancer and hence could be involved in the initiation and/or early stages of prostate cancer development.

B. Functions of TSPY in Health and Diseases

a) TSPY Expression in Fetal and Adult Testis (Reprints 3, 5, 8)

The human TSPY gene is expressed in fetal, neonate and adult testes [1, 18, 19], primarily located in prespermatogonial cells of fetal, as early as 15 week gestation (earliest stage examined), and neonate testes, parallel to several germ cell markers, i.e. placental/germ cell alkaline phosphatase (PLAP), the stem cell factor receptor, c-KIT, and the transcriptional regulator and marker of pluripotency, OCT4, associated with both normal immature and malignant cells in CIS/ITGCN [18]. In adult, TSPY is expressed most abundantly in spermatogonia and round spermatids. The embryonic expression of TSPY is significant because germ cell tumors are postulated to have their origins in primordial or early embryonic germ cells that have acquired/primed with mutational events, e.g. inappropriate expression of certain growth regulatory genes, during their differentiation, leading to development of germ cell tumors later in life [20]. Hence, its embryonic expression in early germ cells raises the possibility that it might play a role in the early predisposition event(s) in TGCTs.

The rat harbors a single functional *Tspy* gene on its Y-chromosome, the human and rat genes differ in their expression patterns, suggesting that they might serve different or variant functions in the testis. Transcripts of *rTspy* were first detected in the testis of 28 days old rats, at which time the first wave of meiotic division was occurring. The rTspy protein was initially detected in stage-9 elongating spermatids and peaked at stage-13 spermatids in adult testis, but not in spermatogonia, unlike the expression pattern of the human TSPY gene. Using a GST pull-down assay, we demonstrated that rTspy could bind to the core histones H2A, H2B, H3 and H4. Rat Tspy co-localized with the histones in the cytoplasm of selected elongated spermatids. Our results suggest that the rTspy may play critical roles as a histone chaperone during maturation of the elongating spermatids in the rat testis.

b) Existence of Polymorphic Human TSPY Proteins (Reprints 1, 8)

Studies in both our laboratory and that of Jörg Schmidtke demonstrated three specific types of transcripts from the tandem repeats of the TSPY gene [1, 19, 21]. The predominant transcript, designated as type 1 transcript, encodes a protein of 308 amino acids [12]. All other types of transcript encode slightly polymorphic TSPY proteins that harbor a conserved (SET/NAP) domain homologous to that shared by other cyclin B binding proteins [12].

To determine which types of TSPY transcript exist in normal and cancerous tissues, we have recently examined the transcript populations of TSPY in normal and tumor samples of prostate and testis (38). Our results showed that 3 types of alternative processing of the TSPY transcripts involving the first exon in which a cryptic donor site at sequence immediately following amino acid residue #29 is used to splice into three different acceptor sites within exon 1 and exon 2. The first two variants splice into sequence preceding amino residue #118 and #135 respectively in exon 1 while the third one splices into that preceding amino residue #170 in exon 2. These cryptic RNA splices result in in-frame deletions of 93, 110 and 145 amino acids from their respective ORFs, and are designated as variant Exon1A (H71), Exon1B (H104) and Exon1C (H109) respectively. Other variant transcripts skip off the small introns, 3 and/or 4, in the RNA processing, resulting in altered reading frames beyond the additional sequences from the intron(s) and slightly different proteins at the carboxyl ends. These latter types are relatively rare. All encoded proteins, however, harbor a whole or partial SET/NAP domain in their respective ORFs. The shortened versions (e.g. Exon1A, Exon1B and Exon1C) were more prevalent in the mostly cancerous prostatic samples than the normal samples. These observations could be significant if the respective TSPY isoforms serve different/variable biological functions.

c) Over-expression of TSPY Potentiates Cell Proliferation and Shortening of G_2/M (Reprint 4)

As discussed above, many of the cyclin B binding proteins are involved in cell cycle regulation. We postulate that TSPY interacts with type B cyclins and exerts regulatory functions in male germ cell proliferation and meiotic division in the testis [11, 19]. When it is aberrantly expressed in dysgenetic germ cells (as in streaked gonads of XY females and testicular dysgenesis syndrome) and epithelial cells in prostate incapable of entering male meiosis, it may potentiate a somatic cell proliferation, contributing to the multi-step oncogenic process(es). To examine this possibility, we have utilized the Tet-Off transactivation system [22] to manipulate the expression of a transfected TSPY gene in cultured HeLa and NIH3T3 cells. HeLa cells are human female cells (without the Y chromosome) while NIH3T3 cells are mouse cells whose Tspy gene is non-functional [23, 24]. In this system, both HeLa and NIH3T3 cells harbor a constitutively expressed transactivator gene (CMV-tTA) that can transactivate a responder gene, consisting of a responsive promoter and a bicistronic construct (TIG-TSPY) coding for both TSPY and the green fluorescent protein (EGFP). In the presence of doxycycline, such transactivation is abolished thereby repressing

TSPY and EGFP expression. Hence, one can compare the effects of over-expression of the target genes between two identical cell populations cultured in the absence or presence of the antibiotic.

Cells transfected with a functional TIG-TSPY construct and cultured in selective media without doxycycline gave consistently higher a number of colonies than that of the same cells selected with media containing doxycycline while the vector alone did not show such effect. Although preliminary in nature, under identical conditions the abbreviated forms of TSPY (i.e. Exon1A-H71 and Exon1C–H109) showed higher transfection efficiencies than those with full-length proteins (i.e. Type 1 & 2). Conversely, those harboring truncated carboxyl termini of the protein, due to skipping of introns 3 (H80) or 4 (H30), gave relatively lower transfection efficiencies. The latter forms of TSPY harbor only partial SET domain. Significantly, transfection of TSPX gene in the same vector resulted in much lower colonies when the TSPX transgene was expressed than when it was repressed. Previous studies demonstrated that over-expression of TSPX [25], as well as SET [26], in cultured cells arrested cell growth, primarily at G_2/M stage [25, 26].

Cell proliferation studies demonstrated that both HeLa and NIH3T3 cells had 30-40% higher proliferative activities than repressed cells, parental cells and cells transfected with vector alone. Hence, over-expression of TSPY potentiates cell proliferation in vitro. Flow cytometry analysis revealed that there were fewer cells at G_2/M stage when TSPY was highly expressed than when it was repressed. Cells released from synchronization at G_1/S boundary progressed through the cell cycle faster, i.e. reaching G_2/M and G_1 stages faster, when TSPY is expressed at high levels than those with repressed TSPY transgene. Hence, TSPY might mediate a rapid transition through G_2/M (and perhaps G_1 phases), thereby shortening duration of the cell cycle for the over-expressor cells. These results suggest that TSPY possesses contrary properties in cell cycle regulation with those of TSPX that, when over-expressed, arrests cells at G_2/M stage [25]. Such rapid transition through G_2/M could increase genomic instability and mutations of the cells, since less time is available for proper chromatin packaging and activation of G_2/M checkpoints, essential for proper DNA repair and mitotic division.

d) TSPY Up-regulates Pro-growth Genes and Represses Inhibitors for Cell Proliferation (Reprint 4)

To determine the effects of TSPY over-expression in the transcription profiles of HeLa cells, we have collaborated with Drs. Wai-Yee Chan and Tin-Lap Lee, Laboratory of Clinical Genomics, National Institute of Child Health and Human Development, NIH in analyzing the gene transcription profiles of HeLa cell populations harboring either a stably integrated TIG-TSPY or TIG vector alone using the Affymetrix Human GeneChip microarray (U133 plus 2.0). The chip contains over 47,000 transcripts and variants, including 38,500 well-characterized human genes. The experiments were conducted with 2 samplings of the respective cell populations. To obtain both expression and functional alterations conferred by TSPY, genes differentially expressed in the HeLa cells harboring the TSPY transgene were first analyzed by the Significance Analysis of Microarrays and exported with SAMSTER to CLUSTER and TREEVIEW programs [27-29]. The biological functions of the differentially expressed genes were then analyzed with the Gene Ontology Tree Machine (GOTM) program [30]. Our results showed that TSPY and a limited number of genes were differentially expressed in the two cell populations. TSPY was expressed at the highest level while others were consistently affected at modest levels. These findings were consistent with the fact that the two HeLa cell populations were almost identical, except the high TSPY expression in one and not the other. The GOTM program identified cell cycle regulation ad the biological process whose genes were consistently altered in cells with constitutive TSPY expression. Table 1 lists 25 cell cycle related genes whose expression was either up- or down-regulated by over-expression of TSPY. Among the up-regulated genes were several oncogenes (epidermal growth factor receptor

(ERBB), and members of the WNT and RAS oncogenes), growth factors (PDGFC, EGF-related, ANKRD15, RGC32, NANOS1), cyclin D2, and a co-factor for the hypoxia inducible factor 1A (EP300), an apoptosis inhibitor (GSPT1) and an antigen (CD24) highly expressed in small cell lung carcinoma. The down-regulated genes included an inhibitor for CDK4/CDK6, transforming growth factor β3, a pro-apoptotic factor (IGFB3), and an inhibitor of MAP kinases (dual specificity phosphatase 5). In particular, the CCND2 gene (encoding cyclin D2) resides on chromosome 12p that is frequently amplified and expressed at high levels in TGCTs. Cyclin D2 complexes with CDK4 or CDK6 to mediate G1/S transition and promote cell proliferation. CCND2 (cyclin D2) was up regulated in the HeLa cells over-expressing TSPY. Conversely, CDKN2B encodes an inhibitor (INK4B) of cyclin D2 activities by acting on CDK4 and CDK6. It was down regulated in the same cells. Loss of INK4 kinase inhibitors has been postulated to be important for progression from CIS to invasive germ cell tumors [7, 31].

To validate the microarray data, we had selected 4 up-regulated (RGC32, PDGFC, WNT5A and CCND2) and 4 down-regulated (CLU1, IGFBP3, TIMP1 and SPARC) genes and performed quantitative RT-PCR analysis with specific primers from the respective cDNA sequences using the ABI-7900HT real time PCR machine. Our results confirmed the up-regulation of the RGC32, PDGFC, WNT5A and CCND2 genes and down-regulation of the CLU1, IGFBP3, TIMP1 and SPARC genes in HeLa cells over-expressing TSPY as compared those cells harboring the vector alone. Although the relative ratios of up and down regulation of the respective genes seem to be higher by quantitative RT-PCR analysis, these observations support the notion that over-expression of TSPY up-regulates pro-growth genes and represses inhibitors for cell proliferation.

e) Over-expression of TSPY promotes tumor growth in nude mice (Reprint 4)

The in vivo effects of TSPY expression were studied by tumorigenicity assays in nude mice. Both HeLa and NIH3T3 cell clones harboring either the bicistronic responder gene (TIG-TSPY) or the vector (TIG) alone were inoculated subcutaneously on the flanks of nude mice. Other animals were inoculated with the parental cells alone. They were fed either with or without doxycycline in their drinking water. Six animals were used for each criteria point. Tumor sizes were measured weekly for 7 weeks. Results showed that tumors grew significantly faster in nude mice inoculated with HeLa Tet-Off cells harboring an over-expressed TSPY gene than in those harboring the vector alone (including EGFP). Nude mice harboring the same HeLa cells whose TSPY gene is repressed by feeding doxycycline-containing drinking water showed the same rate of tumor growth as those in hosts inoculated with HeLa cells transfected with vector alone or the parental cells. Significantly, the NIH3T3 Tet-Off cells are not tumor cells. Under normal conditions, no tumor should result from such inoculation. Mice inoculated with NIH3T3 cells over-expressing a TSPY gene, small tumors were observed in 5 of the 6 mice in this group fed with normal drinking water. Results from this study suggest that over-expression of TSPY potentiates cell proliferation and promotes tumor growth in nude mice.

f) TSPY Binds to Type B Cyclins through its SET/NAP Domain

TSPY harbors the SET/NAP domain present in numerous cyclin B binding proteins, involved in cell cycle regulation. Its effects on cell cycle progression suggest that such cyclin B binding domain might indeed play a role in its function. To confirm its cyclin B binding properties, we performed various pull-down assays with GST-fusion baits and in vitro transcribed and translated proteins. Initial analysis showed that ^{35}S-labeled full-length TSPY and abbreviated isoforms were pulled down with similar efficiency by a bait of GST-human cyclin B1 protein (Figure 3A), but not those with cyclin A or GST alone. To identify the specific domain(s) of TSPY

that interact with cyclin B1, various deletion mutants were generated (Figure 3B and C) and used for syntheses and labeling of various truncated TSPY proteins. GST pull-down assays with GST-

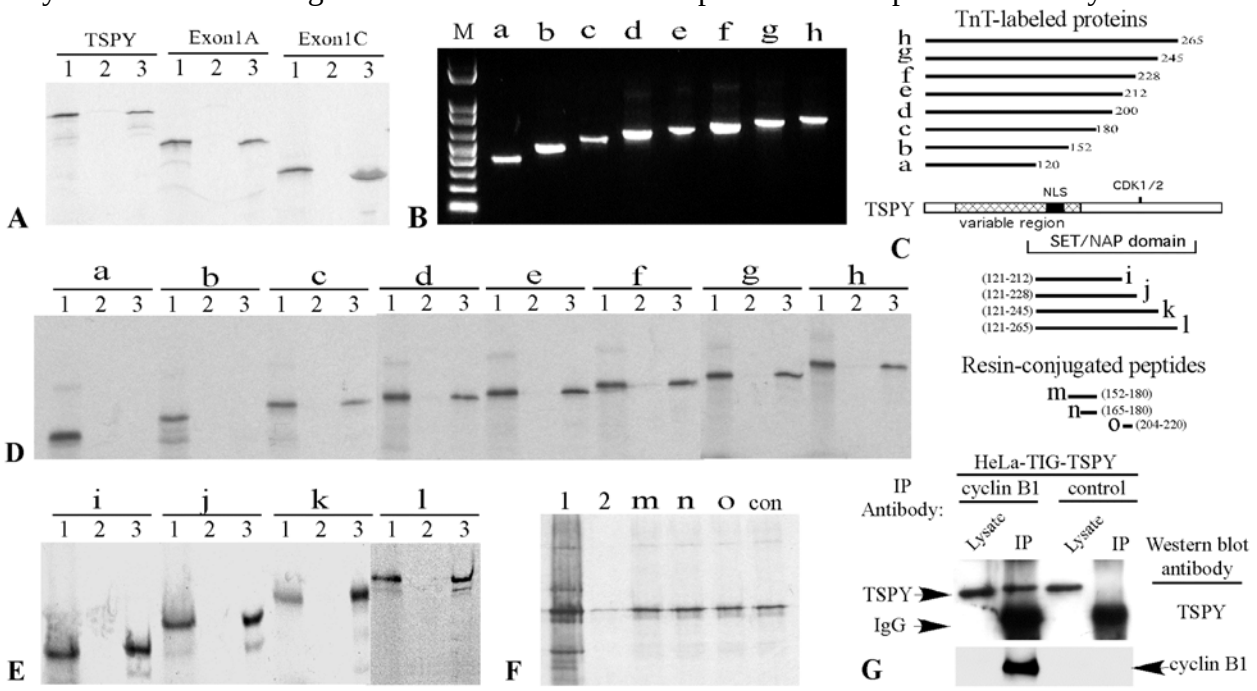


Figure 3. Interactive studies of TSPY and cyclin B1. A) Full-length and variant Exon 1A and 1C were labeled by in vitro transcription-translation reactions and analyzed with pull-down assays using GST or GST-cyclin B1 fusion proteins as baits. All isoforms of TSPY interacted with GST-cyclin B1 fusion protein, but not GST alone. 1=input; 2=GST; 3=GST-cyclin B1 fusion protein. To map the cyclin B1 binding domain in various TSPY deletion mutants were generated (B and C) and analyzed similarly with the pull-down assays (lanes a to h in (D) and lanes i to l in (E). These initial studies mapped the cyclin B interactive domains to residue #120-228 at the SET/NAP domain of the TSPY full-length protein. To further define the cyclin B binding domains, specific peptides were synthesized and conjugated to Sepharose 4B and analyzed with TnT labeled cyclin B1 protein (F). This later study defined two domains, residues #165-180 and #204-220, of the TSPY protein to be responsible interacting with cyclin B1. G) Immuno-precipitation of TSPY-transfected HeLa cell lysate with cyclin B1 and control antibodies, and western blot with TSPY (top panel) and cyclin B1 (lower panel) antibodies. TSPY interacts with cyclin B1 in vivo and is co-immunoprecitated with cyclin B from the HeLa cells transfected with a TSPY transgene.

cyclin B1 bait narrowed the cyclin B binding domain to residue #120-228 within the SET/NAP domain (Figure 3C-E). To further define these domains, various peptides corresponding to this region were synthesized and conjugated to CNBr-activated Sepharose 4B and used as baits in pull-down assays with in vitro labeled human cyclin B1. This study further mapped the cyclin B binding domains to two regions at residue #165-180 and #204-220 (Figure 3F). To confirm the in vivo interaction of TSPY-cyclin B1, HeLa cells expressing a TSPY transgene were immunoprecipitated with a human cyclin B1 monoclonal antibody and analyzed with a TSPY polyclonal antibody. The results showed that TSPY was efficiently co-IP with the cyclin B1 antibody. Reprobing the filter with a cyclin B1 antibody confirmed that cyclin B1 was indeed enriched in the immunoprecipitated sample, but not with a control antibody (Figure 3G), confirming that TSPY indeed interacts in vivo with cyclin B1 in the HeLa cells.

g) TSPY Interaction Enhances the Phosphorylation Activities of Cyclin B-CDK1 Complex

Previous studies of TSPX (CDA1) gene suggests that two specific CDK1 phosphorylation sites present at its protein are responsible for its effects on arresting cells at G_2/M stage when it is over expressed [25]. Since one of these phosphorylation sites is conserved in TSPY (at the threonine residue #233) and TSPY possesses contrasting properties to those of TSPX, it will be important to determine if TSPY can be phosphorylated by a cyclin B1-CDK1 complex and what effects TSPY binding to cyclin B might have on CKD1 kinase activities. Using a phosphorylation assay, we demonstrated that TSPY was indeed phosphorylated by an activated human cyclin B1-CDK1 complex (CalBiochem Inc.) in the presence of ^{32}P-γ-ATP (Figure 4A). The efficiency of phosphorylation on TSPY, however, was about 8-10 folds slower than that of cyclin B1-CDK1 on histone H1. To determine the effects a TSPY interaction on cyclin B1-CDK1 activities, the phosphorylation of histone H1 was analyzed similarly in the presence of increasing amount of TSPY. The results showed that addition of TSPY in the reaction mixtures increased the kinase activities of the cyclin B1-CDK1 complex (Figure 4B). These findings suggest that TSPY might exert its pro-growth effects by interacting with type B (mitotic and meiotic) cyclins and enhancing the cyclin B-CDK1 kinase activities. Since cyclin B must execute various critical functions, including interaction and activation of CDK1 and CDK2 and phosphorylation of target proteins, in mediating the transition of the cell through G_2/M phase, the TSPY enhancement of such activities, therefore, might expedite such cyclin B functions, resulting in a shortening of the G_2/M phase, as observed in HeLa cells over-expressing a TSPY transgene. Hence, aberrant TSPY expression in the prostate might exert a similar pro-proliferation and/or oncogenic function in the epithelial cells.

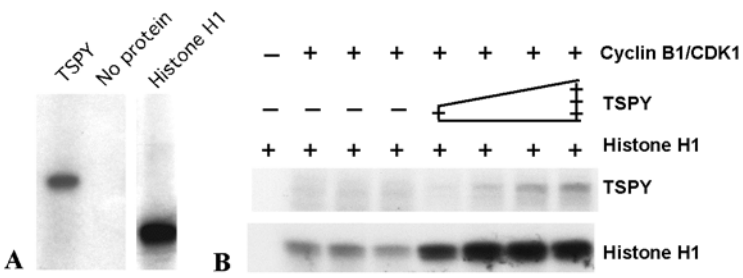
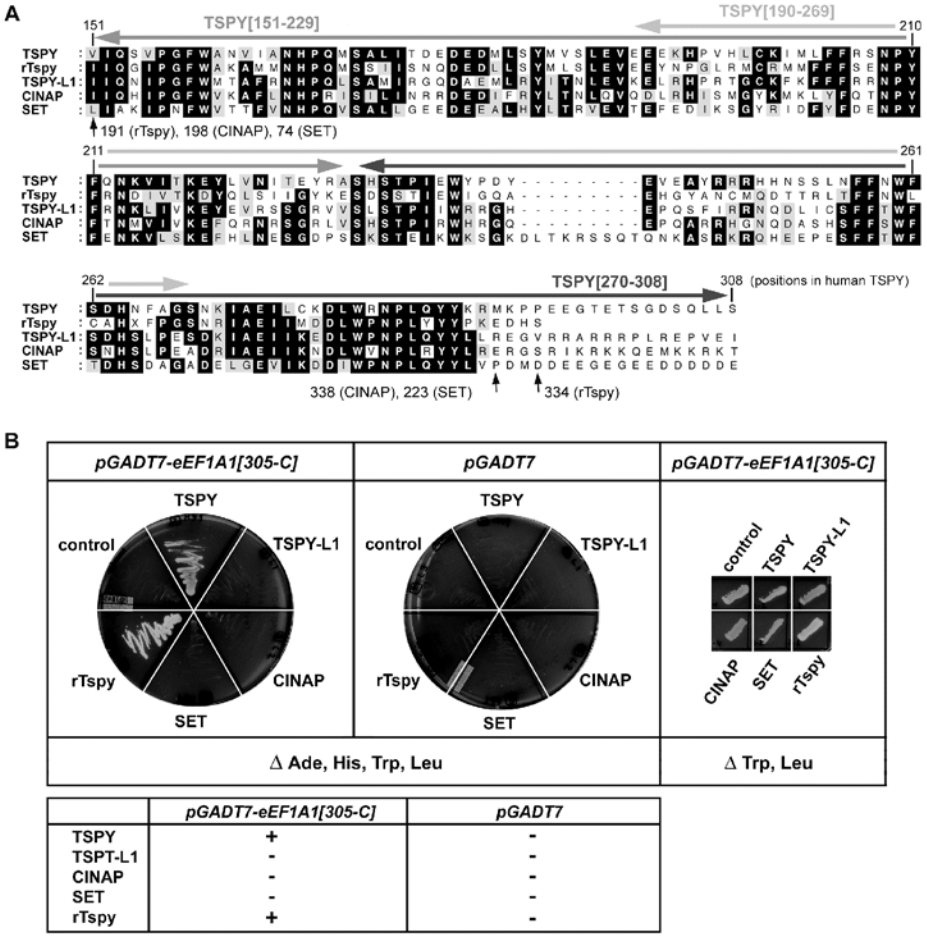


Figure 4. TSPY interacts with cyclin B1 and enhances the cyclin B1-CDK1 kinase activities. A) TSPY was phosphorylated by a cyclin B1-CDK1 complex, abide ~10 folds slower than that of histone H1. B) The phosphorylation of histone H1 was enhanced proportionally with addition of TSPY proteins in the kinase reactions, while TSPY was simultaneously phosphorylated. TSPY and histone H1 autoradiograms were obtained by different exposure times.

h) Identification of the eukaryotic protein elongation factor 1 alpha 1 (eEF1A1) as an interactive partner for TSPY

To elucidate the molecular mechanism(s) responsible for TSPY functions, we have performed a yeast two-hybrid screen of a mouse fetal gonadal cDNA library using the TSPY SET/NAP domain as bait. Forty clones out of 130 well-grown blue colonies were randomly selected for DNA sequencing. One of these clones encodes the C-terminus region (residues 305-462) of eEF1A1, the eukaryotic protein elongation factor 1 alpha 1. Further analysis of all 130 colonies by PCR showed that 4 out of 130 colonies harbored the eEF1A1 cDNA. AH109 yeast cells transformed with either pGBKT7-TSPY[151-308] or pGADT7-full length eEF1A1 showed negative growth on selection medium (data not shown). eEF1A1 is one of two isoforms (eEF1A1 and eEF1A2) of translation elongation factor alpha (eEF1A). eEF1A1 is ubiquitously expressed while eEF1A2 is differentially expressed primarily in postnatal stages [32]. eEF1As are mostly localized in cytoplasm and play significant roles in recruit of tRNA in protein synthesis and nuclear export [33, 34].

Since the SET/NAP domain of TSPY shares significant homology with those of other proteins of the TSPY/SET/NAP1 family (Figure 5A), we further explored possible interactions between eEF1A1[305-462] and other TSPY/SET/NAP1 family members, such as TSPY-like1, CINAP/TSPX, SET and rat Tspy using the yeast two-hybrid strategy. Our results showed that only the human TSPY[151-308] and rTspy[185-334] interacted with eEF1A1[305-462] in yeast (Figure 5B).



	pGADT7-eEF1A1[305-C]	pGADT7
TSPY	+	-
TSPT-L1	-	-
CINAP	-	-
SET	-	-
rTspy	+	-

Figure 5. The SET/NAP domain of human TSPY and eEF1A1 interact in yeast. A, amino acid sequence alignment of SET/NAP domains of human TSPY/SET/NAP1 family proteins and rat Tspy (rTspy). The amino acid residues highlighted in black are conserved, and those regions with conserved chemical properties are shown in grey. The regions of TSPY used in GST pulldown assays are indicated by gray lines. The MacVector Sequence Analysis Program (MacVector Inc.) was used for generation of the sequence alignment. B, yeast colonies that grew on plates lacking tryptophan and leucine (DTrp, Leu) were examined for growth on adenine- and histidine-deficient plates (DAde, His, Trp, Leu). Growth in the absence of leucine indicates the presence of pGADT7 or pGADT7-eEF1A1[305-C], whereas growth in the absence of tryptophan indicates the presence of pGBKT7 containing the indicated derivatives of TSPY/SET/NAP1 family proteins. Interactions between proteins are shown by the ability to activate the ade2 and his3 reporter genes. Abbreviations; control, pGBKT7 vector alone; TSPY, pGBKT7-TSPY[151-308]; TSPY-L1, pGBKT7-TSPY-like1[full-length]; CINAP, pGBKT7-CINAP[198-338]; SET, pGBKT7-SET/TAF-Ib[74-223]; rTspy, pGBKT7-rat Tspy[185-334].

i) In vitro mapping of the interactive domains between TSPY and eEF1As

In vitro GST pull-down assays were used to confirm the interaction between TSPY and eEF1A and to delineate the respective interactive domains. An interaction was observed between eEF1A1[305-462] and GST-TSPY[full-length], but not with GST alone (Figure 6*B*). To map the interactive domains of TSPY responsible for its eEF1A1 binding, a series of GST-TSPY truncated mutants, TSPY[151-229], GST-TSPY[190-269] and GST-TSPY[230-308], were generated and used as baits in respective pulldown assays with ^{35}S-labeled eEF1A1[305-462]. Both GST-TSPY[190-229] and GST-TSPY[230-308], which contain the highly conserved sequences with other TSPY/SET/NAP1 family (Figure 5*A*), could bind eEF1A1[305-462] effectively (Figure 6*C*, lanes 3 and 5 in top). TSPY mutants lacking either the region [151-189] or [270-308] showed no or faint binding to eEF1A1[305-462] (Figure 6*C*, lane 4 in top).

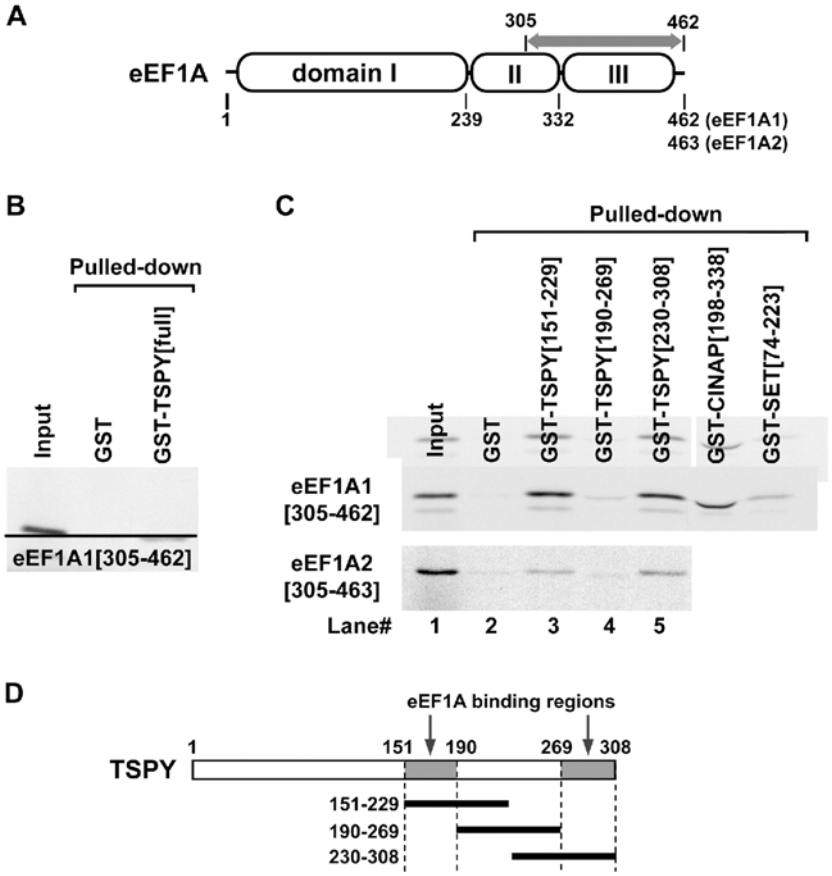


Figure 6. The C-terminus region of eEF1A interacts with the SET/NAP domains of TSPY and CINAP/TSPX in vitro. A, domain structure of the full-length eEF1A. The positions of amino acid residues were derived from computer alignment with yeast eEF1A (GenPept accession number; NP_009676) using the MacVector program. The region harbored in the cloned pGADT7-cDNA library is indicated by the bold bar (amino acid residues 305-462 of eEF1A1). The regions corresponding to the eEF1Ba-, GTP-, and aa-tRNA-binding sites of yeast eEF1A are shaded in gray; [residues 22-167] and [252-322], respectively). B, GST alone or the indicated GST-fusion proteins immobilized on glutathione-agarose beads were incubated with 35S-labeled eEF1A1[305-462]. After pulldown, the bound proteins were resolved by SDS-PAGE and detected by autoradiography. The C-terminus region of eEF1A1[305-462] bound specifically to full-length TSPY. Input lane represented 8% of the peptides used for the pulldown assay. C, GST pulldown was performed as in B with the indicated GST-fusion proteins and 35S-labeled eEF1A1[305-462] or eEF1A2[305-463]. The TSPY regions used in the binding assays with eEF1A are illustrated as shown at the bottom.

The analogues, eEF1A1 and eEF1A2, share >90% identity and have essentially the same function in protein translation [35]. To explore the possibility of a TSPY and eEF1A2 interaction, the eEF1A2[305-463] expression vector pcDNA-eEF1A2[305-463]-V5 was generated, *in vitro* translated with ^{35}S-methionine, and used similarly in the GST pulldown assays with a series of GST-TSPY mutants, as described above. The results showed that eEF1A2[305-463] was capable of binding to TSPY at specific domains as those for the eEF1A1 (Figure 6*C*, lanes 3 and 5 in bottom), as summarized in Figure 6*D*.

Although, eEF1A1 did not show any significant in vivo binding to the conserved SET/NAP domains of other members of the TSPY/SET/NAP family, we explored the possibility they might interact in vitro in GST pulldown assays. Our results showed that GST-SET[74-223] (Figure 6*C*, lane 7)and GST-CINAP[198-338] (Figure 5*C*, lane 6) bound readily with eEF1A1[305-462]. Our observation is consistent with those described in a recent report on SET-binding proteins analyzed by proteomics strategies [36]. Currently, we are uncertain of these differences between in vivo yeast two-hybrid and in vitro GST pulldown approaches on the interactions between eEF1A1 and SET/CINAP. Interestingly, both CINAP/TSPX and SET are localized in nuclei, whereas eEF1A is restricted in cytoplasm. Further cellular and physiological analyses will be required to elucidate the significance, if any, of the interaction among these molecules.

j) TSPY co-localizates and interacts with eEF1A and forms homodimers in mammalian cells.

To confirm the interaction between TSPY and eEF1A in mammalian cells, immunofluorescence was performed on COS7 cells transfected with TSPY, and FLAG-eEF1A1 or eEF1A2-V5 expression vectors. eEF1As exhibited mostly cytoplasmic staining (Figure 7*A* and 7*B*, FLAG-eEF1A1 or eEF1A2-V5 respectively) while TSPY were localized in both nucleus and cytoplasm. The cytoplasmic TSPY exhibited an almost identical staining pattern with both eEF1As (Figure 7A and 7B, TSPY), as revealed on merged the images of TSPY and eEF1As (Figure 7A and 7B, yellow dots in the merged images). To confirm their interactions in the transfected cells, total cell lysates were co-immunoprecipitated with an anti-FLAG antibody. The precipitates were then analyzed by Western-blot using anti-TSPY antibody. The result showed that TSPY was pulled-down with FLAG-eEF1A1, but not in the absence of FLAG-eEF1A1 (Figure 7C), thereby confirming an interaction between TSPY and eEF1A in transfected COS7 cells.

Recently, the atomic structure of the functional domain of the human SET protein had been elucidated [37]. The SET protein forms homodimer through their α2-helices locating on N-terminal region [37]. Using the web-based Protein Homology/analogy Recognition Engine (Phyre) program (http://www.sbg.bio.ic.ac.uk/phyre/html/index.html) [38], we had analyzed the TSPY structure, using the SET atomic structure as a model. Our analysis identified two long α-helices (α1 and α2) at residues 52-95 and 105-141 of TSPY, analogous to those of SET. To explore the possibility that TSPY indeed could form homodimers, the interaction between the full-length TSPY and an abbreviated form, FLAG-TSPY[76-150], was examined by co-immunoprecipitation, similarly as described above. Our results show that the full-length TSPY was pulled-down with FLAG-TSPY[76-150] (Figure 7D), suggesting that TSPY could form homodimers through its N-terminal domain.

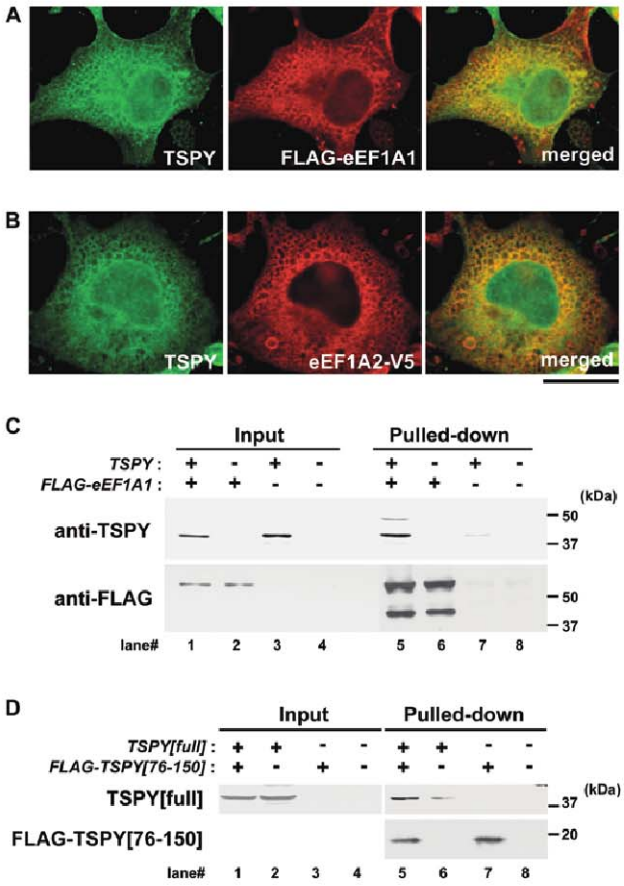


Figure 7. TSPY colocalized and interacted with eEF1A in mammalian cells. A, COS7 cells were transiently cotransfected with TSPY and FLAG-tagged eEF1A1 expression vectors. Cells were examined 2 days later by indirect immunofluorescence for the cellular distribution of TSPY and eEF1A1 using anti-TSPY and anti-FLAG antibodies. B, the cellular distribution of TSPY and eEF1A2 in COS7 cells was analyzed as in A with anti-TSPY and anti-V5 antibodies. TSPY and eEF1A were well colocalized in the cytoplasm of transfected cells, resulting in a yellowish color in the merged images. Scale bar= 25 mm. C, COS7 cells were cotransfected with the indicated combinations of plasmids (TSPY, pCS2-TSPY; FLAG-eEF1A1, pFLAG-eEF1A1; -, appropriate empty vector). Lysates were collected at 2 days and examined by Western blot for expression of TSPY (input, top) and FLAG-eEF1A1 (input, bottom). Immunoprecipitation was performed with anti-FLAG antibody to detect an interaction between TSPY and eEF1A1. The interacting proteins were analyzed by Western blot using anti-TSPY and anti-FLAG antibodies (pulled down). TSPY was specifically pulled down with FLAG-tagged eEF1A1 (lane 5). D, COS7 cells were cotransfected with the indicated combinations of plasmids (TSPY, pCS2-TSPY; FLAG-TSPY[76-150], pFLAG-TSPY[76-150]; -, appropriate empty vector). Interaction between TSPY and TSPY[76-150] was analyzed by immunoprecipitation as described in C. Full-length TSPY was significantly co-immunoprecipitated with FLAG-tagged TSPY[76-150] (lane 5, arrow). Open arrowhead indicates the IgG bands originated from anti-FLAG antibody used in immunoprecipitation.

k) TSPY stimulated reporter protein synthesis in HEK293 cells

Since eEF1A is an important component of the cellular translational machinery, by interacting with eEF1A, TSPY could affect the overall protein synthesis in the cell. To examine this possibility, we had characterized the effect of ectopic TSPY expression on protein synthesis, in terms of the levels of a luciferase reporter activity in cultured cells. HEK293 cells were transiently co-transfected with either pCS-TSPY or pCS-hEEF1A1 construct, and combination thereof (0 - 0.4 mg/well) and *pRL-TK* (5 ng/well) in quadruplicates. The inclusion of the pRL-TK plasmid was designed to provide low to moderate levels of luciferase expression under the control of herpes

simplex virus thymidine kinase promoter. Forty-eight hours after transfection, the cells were then lysed and analyzed for luciferase activity. When *pRL-TK* was co-transfected with the human eEF1A1 expression vector, *pCS-hEEF1A1*, the transfected cells showed higher luciferase activity (Figure 8, column 6 and 7) than control (Figure 8, column 1), suggesting that increase expression of eEF1A enhanced the protein synthesis in HEK293 cells. To evaluate the effect of TSPY on protein synthesis, pCS-TSPY was co-transfected with the luciferase reporter. Our results demonstrated that ectopic expression of TSPY stimulated luciferase activities in a dosage dependent manner (Figure 8, column 1 to 5), presumably proportional to the indirect effects of TSPY on the synthesis of this reporter. Significantly, TSPY could further enhance the stimulation of eEF1A1 on the luciferase expression in the transfected cells (Figure 8, column 8 and 9). These observations suggest that TSPY could enhance the protein production via its interaction with eEF1As, components of the protein translational machinery in the cell.

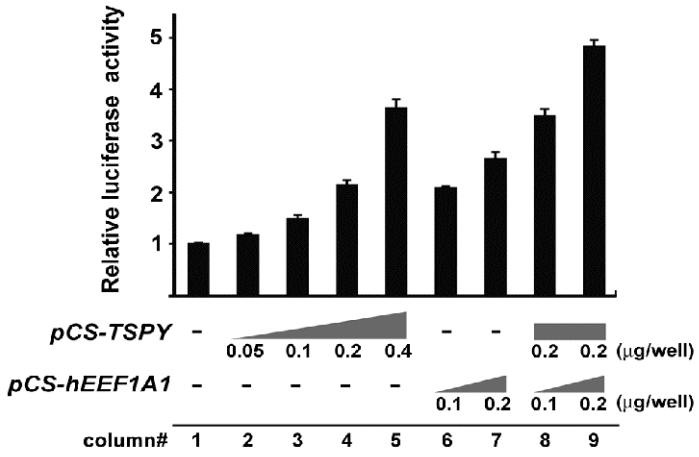


Figure 8. Stimulation of the model protein synthesis by eEF1A and TSPY. HEK293 cells were cotransfected with pRL-TK (5 ng/well) in the presence of various amounts of pCS-TSPY and/or pCS-hEEF1A1 as indicated in the figure. Total DNA was adjusted with empty vector (pCS2-plus). Luciferase activities were measured 2 days after transfection. The indicated relative activity is based on the luciferase activity of the control (0 mg/well of both pCS-TSPY and pCS-hEEF1A1). The data represent the means ± SE of four individual experiments. Lower panel shows the expression level of TSPY and a-tubulin as an internal control.

TASK 2. TO CORRELATE TSPY OVER-EXPRESSION WITH PROSTATIC ONCOGENESIS IN TRANSGENIC MICE

a) Expression of TSPY in a Tissue Recombination Model of Prostate Cancer

In adult prostate, homeostasis is maintained via reciprocal stromal and epithelial interactions [39, 40]. During carcinogenesis, following genetic damage to the epithelium, such reciprocal interactions are disrupted, favoring proliferation for the epithelial cells. Recently, excellent tissue recombination models of stromal-epithelial interactions have been established primarily in Dr. Gerald Cunha's laboratory at UCSF and used to study the hormonal and genetic determinants involved in normal and malignant prostatic growth. One such model utilizes an SV40 T antigen-immortalized non-tumorigenic human prostatic epithelial cell line, BPH-1, and stromal cells from various sources [39, 41, 42]. The BPH-1 cells were initially developed by Dr. Simon Hayward, a Collaborator in this project, while he was in Dr. Cunha's laboratory at UCSF. In general, such tissue recombinants with normal stromal cells, when transplanted to the kidney capsule of nude mice, produce solid branched epithelial cords and ductal structures with a benign histology. In testosterone and estradiol ($T+E_2$) treated hosts, such recombinants develop focally invasive carcinomas. Similarly, when human carcinomas-associated fibroblasts (CAFs) from prostate cancer patients are used as stromal donors, large aggressively malignant tumors develop [41]. The oncogenic processes are dependent on genetic damage to the epithelium. Such damage can result from specific genetic targeting, such as the knockout of the RB tumor suppressor gene [43]; or by

the more general genetic damage caused, for example by the expression of viral oncogenes [42]. These studies emphasize the importance of stromal microenvironment, genetic and hormonal influence in the carcinogenic transformation of the epithelial (BPH-1) cells.

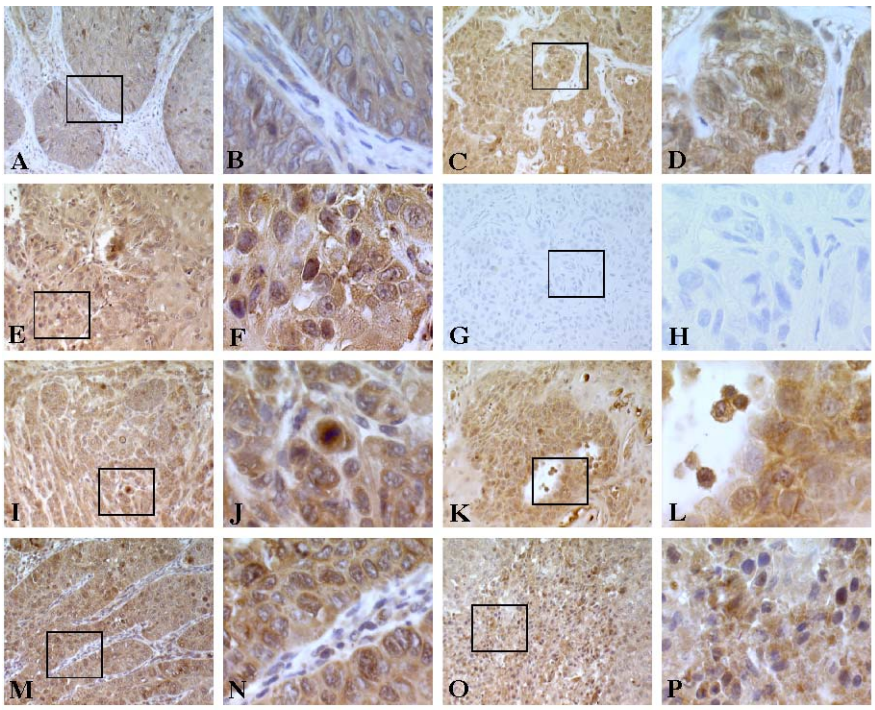


Figure 9. TSPY expression in BPH-1-rUGM prostatic tissue recombinants. A-B) Normal; C-D) T+E2 treated; G-H) staining without primary antibody, negative control of C-D; E-F) BPH-1-CAF recombinant; and derivative BPH-1 lines from BPH-1-CAF recombinants showing oncogenic growth with rUGM (without CAF) recombination. I-J) BPH-1 $^{CAFTD-03}$; K-L) BPH-1 $^{CAFTD-04}$; M-N) BPH-1 $^{CAFTD-05}$; O-P)BPH-1 $^{CAFTD-08}$. Boxed areas are enlarged in corresponding figures on the right .

In collaboration with Dr. Simon Hayward, now at Vanderbilt University, we have examined the TSPY expression in recombinant transplants consisting of either BPH-1 and rat urogenital sinus mesenchyme (rUGM) or BPH-1 and CAFs. Our results demonstrated that TSPY proteins were expressed only at low levels in the epithelial cells (i.e. BPH-1) in the benign BPH-1+rUGM recombinants (Figure 9A, B) and significantly in the tumor cells induced by $T+E_2$ treatment with the hosts (Figure 9C, D). For the BPH-1 and CAFs recombinants, TSPY is widely expressed and is localized in the carcinoma and cancerous BPH-1 cells (Figure 9E, F). Several BPH-1 derivative lines (Figure 9I–P) have been obtained from sequential grafting of the tumor cells from the initial BPH-1/CAF recombinants. When they are recombined with normal rat UGM, these BPH-1 derivative lines give rise to prostatic carcinoma without the needs of CAF nor hormonal treatment. Hence, they have been selected and sequentially enriched for acquiring additional mutational steps towards the spontaneous oncogenic phenotypes. TSPY stained positively with these tumor cells (Figure 9I-P). These results are in agreement with those observed in $T+E_2$ induced prostate cancer in the Noble rats and further support the postulation that TSPY participates in prostatic oncogenesis. The abundant expression of TSPY in the CAF-induced BPH-1 derivative cells in these recombinants suggests that TSPY activation could be one of the events leading to the tumorigenic properties of these derivative BPH-1 cells. If TSPY is involved in oncogenesis in these models, the observations also suggest that TSPY might be needed to maintain an oncogenic phenotype for such tissue recombinants.

b) TSPY Expression in Prostatic Hyperplasia of Transgenic Mice

Transgenic mice harboring a modular TSPY gene directed by the human kallikrein 2 (HK2) promoter [44, 45] show an epithelial cell-specific expression pattern of the human transgene (Figure 10 A-K). Hyperplasia/PIN could be observed in the prostates of a few animals at 8-12 months.

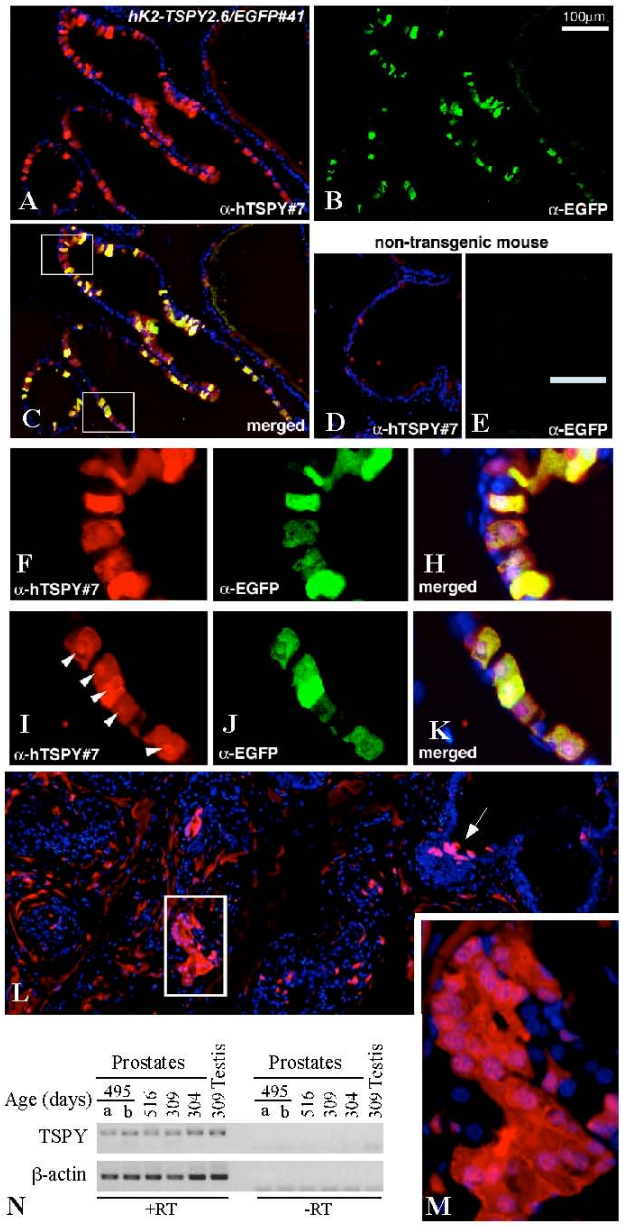


Figure 10. TSPY transgene expression in prostatic epithelia induces hyperplasia in transgenic mice. A-K) TSPY-EGFP biscistronic transgene directed by HK2 promoter is expressed specifically in epithelial cells in the prostates of transgenic mice. L-M) Old transgenic mice harboring a 12-kb human TSPY transgene expressed TSPY in their prostates, resulting in hyperplasia. Arrow indicates a hyperplasic area, similar to boxed area (magnified in M). N) RT-PCR analysis confirmed TSPY transgene expression in the old mice.

TSPY is abundantly expressed in the hyperplasic cells. Similarly, transgenic mice harboring a 12-kb human Y-DNA containing the TSPY structural gene showed a normal testicular expression of the transgene and no expression in the prostate, under normal conditions. However, when these mice aged, numerous hyperplasic foci in their prostates could be observed readily. Immunofluorescence demonstrated that the human TSPY transgene was expressed specifically in these hyperplasic cells (Figure 10L-M). Such transgene expression could be confirmed by RT-PCR analysis (Figure 10N). Since mice harbor a nonfunctional Tspy gene on their Y chromosome and prostatic tumors are not normally observed. The prostatic hyperplasia developed in these TSPY transgenic mice suggests that the human transgene could promote latent lesion or PIN development in its hosts. Although preliminary in nature, these results corroborate with those from clinical samples, suggesting that TSPY expression is closely associated with latent/initial stages of prostatic oncogenesis.

FUTURE DIRECTION

We have successfully implemented the proposed studies in the funded period of this project. The establishment of the Y chromosome gene, TSPY, as a putative oncogene involved in prostate cancer and testicular germ cell tumors clearly positioned this GBY gene in the initiation of such human cancers. Future studies in delineating the molecular mechanisms by which it exerts its oncogenic functions will greatly facilitate developments of diagnostic, therapeutic and preventive strategies for prostate cancer, based on TSPY biology.

KEY RESEARCH ACCOMPLISHMENTS

- Establish TSPY to be the gonadoblastoma gene.
- Demonstrate TSPY to be involved in the initiation of prostatic oncogenesis.
- Demonstrate TSPY expression is proportional to the severity of the prostate cancer.
- Demonstrate Tspy gene expression in a tissue recombinant model of prostate cancer.
- Show the human TSPY transgene is expressed in hyperplasic regions in the prostates of old transgenic mice.
- Demonstrate TSPY effects on cell cycle regulation, i.e. potentiating cell proliferation and shortening the G_2/M phase (leading to potential genomic instability and epigenetic dysregulation of other oncogenes favoring oncogenesis).
- Demonstrate TSPY effects on protein synthetic machinery via its interaction with another oncoprotein, the elongation factor 1A.

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CONCLUSION

We have now established strong evidence supporting TSPY to be the gene for GBY, the only oncogenic locus on the human Y chromosome. Its expression in prostate cancer suggests that it might play a significant role in the multistep oncogenic process in prostate cancer. We have examined additional cases of prostate cancer of different clinical grades and demonstrated that TSPY expression is proportional to the severity of the cancer. We demonstrated TSPY expression increased with malignancy of a tissue recombination model of prostate cancer. Significantly, TSPY is especially expressed in phenotypic normal epithelia adjacent to cancerous regions and in latent cancer foci in prostates of elderly individuals without any clinical symptoms. These findings are critically important; suggesting that TSPY could plays cardinal role(s) in the initiation of prostate cancer. This view is also supported by two additional observations. First, TSPY is expressed in the premalignant germ cells that eventually develop into seminomas and nonseminomas, suggesting that TSPY could also participate in early stages of this type of tumors, a parallel scenario to that of the prostate cancer. Second, TSPY is expressed in the hyperplasic regions in the prostates of old transgenic mice, supporting our clinical observations that TSPY might play an important role in the initiation of prostatic oncogenesis.

Our efforts on identifying the molecular mechanisms by which TSPY exerts its oncogenic functions have been extremely successful in studies supported by this project. First, we showed that ectopic expression of TSPY potentiates cell proliferation in vitro and tumorigenicity in nude mice. It up regulates genes important for cell proliferation and growth and inhibits apoptotic genes.

Significantly, genes on the short arm of chromosome 12 are especially up regulated. These genes are postulated to play roles in germ cell tumorigenesis. Further studies identified the elongation factor, eEF1A, as an interactive partner for TSPY. Since eEF1As are considered to be oncogenes in various systems, including breast and prostate cancers, their interactions with TSPY suggest that they might collaborate with TSPY in tumorigenesis of male-specific cancers. Indeed, ectopic expression of TSPY accelerates the protein synthetic machinery in transfected cells. Since tumor cells are actively proliferating, a central feature of being a cancer cell, TSPY effects on protein synthesis suggest that TSPY exacerbates such tumorigenic feature and contributes to cancer development.

SO WHAT

Our results have clearly associated the Y chromosome gene, TSPY, to the early stages of human oncogenesis, particularly on prostate cancer and testicular germ cell tumors. They will form the basis for future developments of diagnostic, therapeutic and preventive strategies for prostate cancer.

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Expression of a candidate gene for the gonadoblastoma locus in gonadoblastoma and testicular seminoma

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Dedicated to Professor Dr. Ulrich Wolf on the occasion of his retirement.

Abstract. The gonadoblastoma locus on the Y chromosome (GBY) predisposes the dysgenetic gonads of XY females to develop in situ tumors. It has been mapped to a critical interval on the short arm and adjacent centromeric region on the Y chromosome. Currently there are five functional genes identified on the GBY critical region, thereby providing likely candidates for this cancer predisposition locus. To evaluate the candidacy of one of these five genes, testis-specific protein Y-encoded (TSPY), as the gene for GBY, expression patterns of TSPY in four gonadoblastoma from three patients were analyzed by immunohistochemistry using a TSPY specific antibody. Results from this study showed that TSPY was preferentially expressed in tumor germ cells of all gonadoblastoma specimens. Additional study on two cases of testicular seminoma demonstrated that TSPY was also abundantly expressed in all stages of these germ cell tumors. The present observations suggest that TSPY may either be involved in the oncogenesis of or be a useful marker for both types of germ cell tumors.

Gonadoblastoma is a rare tumor that arises mostly in the dysgenetic gonads of phenotypic females who harbor some Y chromosome materials in their genome (Page, 1987). The tumor is composed of aggregates of primordial germ cells and sex cord elements resembling immature Sertoli and granulosa cells. These aggregates are surrounded by luteinized ovarian type stroma that may include Leydig or lutein-type cells (Scully, 1953, 1970). Gonadoblastoma has been considered to be an in situ germ cell malignancy from which invasive germ cell tumors can develop (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Heerbst et al., 1999).

The prevalence of gonadoblastoma among XY females had led David Page (1987) to hypothesize the presence of a locus, gonadoblastoma locus on the Y chromosome (GBY), that predisposes the dysgenetic gonads of these sex-reversed individuals to develop such in situ tumors. Page further predicted that the gene(s) encoded by the GBY locus has a normal function in the testis and acts as an oncogene only in the dysgenetic gonad. Using a panel of DNAs from XY females with gonadoblastoma, Page had initially mapped the GBY locus to deletion interval 3 on the short arm and intervals 4B-7 on the long arm of the Y chromosome. Additional studies further sublocalized this locus to a small region consisting of ~1–2 Mb of DNA in deletion intervals 3E–3G proximal to and 4B at the centromere and possibly 5E, a proximal interval on the long arm (Salo et al., 1995; Tsuchiya et al., 1995). Among the genes so far isolated from the human Y chromosome (Vogt et al., 1997; Lahn and Page, 1997; Lau and Zhang, 2000), there are five genes residing on this small region: amelogenin Y (AMELY), RNA binding

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motif Y (RBMY), protein kinase Y (PRKY), protein tyrosine phosphatase PTP-BL related Y (PRY), and testis-specific protein Y-encoded (TSPY). Hence, they are candidates for GBY. AMELY encodes an enamel protein in the tooth buds (Salido et al., 1992). RBMY is a repeated gene with a majority of its functional members residing at interval 6 on the long arm, outside the GBY critical region (Cooke, 1999). It expresses a protein with RNA binding motif in the nuclei of male germ cells. PRKY is a single copy gene coding for a putative cAMP-dependent serine/threonine protein kinase (Schiebel et al., 1997). Both RMBY and PRKY have a homologous gene, RBMX and PRKX respectively, on the X chromosome (Schiebel et al., 1997; Delbridge et al., 1999). PRY is a recently isolated repeated gene family coding for a protein related to the PTP-BL tyrosine phosphatase (Lahn and Page, 1997). Some copies of PRY are present outside the GBY region. TSPY is a repeated gene whose functional members are primarily located in two clusters, TSPYA and TSPYB, within interval 3 (Zhang et al., 1992; Conrad et al., 1996; Vogt et al., 1997) on the short arm and as a single-copy on the proximal region of the long arm (Ratti et al., 2000). TSPY shares tight homology to a family of cyclin B binding proteins, such as the SET oncoprotein and the nucleosome assembly protein (NAP-1) (Tsuchiya et al., 1995; Schnieders et al., 1996), and has been postulated to play a role in directing the spermatogonial cells to enter meiosis (Schnieders et al., 1996; Vogel et al., 1998). Other cyclin B binding proteins have been demonstrated to be involved in the mitotic process, cell proliferation and/or carcinogenesis (von Lindern et al., 1992; Adachi et al., 1994; Altman and Kellogg, 1997; Carlson et al., 1998; Shin et al., 1999). Hence, aberrant or inappropriate expression of TSPY in dysgenetic gonads may play a role in the etiology of gonadoblastoma.

To evaluate the candidacy of TSPY as the gene(s) for GBY, we have performed detailed expression analysis of TSPY in gonadoblastoma and testicular seminoma, or germ cell tumor, using immunohistochemical techniques. Our results demonstrate that TSPY is preferentially expressed in the germ cells of the tumor aggregates in gonadoblastoma and tumor cells at different stages of testicular seminoma. Its expression pattern is very similar to those of cyclin B1 and another cell proliferative marker, the proliferating cell nuclear antigen (PCNA). These findings, hence, support the postulation that TSPY is a significant candidate for GBY, and suggest a possible role of TSPY in the multi-step carcinogenesis of testicular seminoma.

Materials and methods

Patients

Tissue sections were obtained from archival formalin-fixed and paraffin-embedded tumor specimens. All three gonadoblastoma patients had previously been described (Iezzoni et al., 1997; Hussong et al., 1997). At the time of biopsies, Patient #1 was a 15-year old phenotypic female with a unilateral gonadoblastoma at the left gonad. Patient #2 was a 20-year old phenotypic female with bilateral gonadoblastoma. Both patients #1 and 2 have a 45,X/46,XY mosaic karyotype. Chromosome painting analysis on tissue sections of these tumors showed that most tumor cells harbored a Y chromosome while the stroma showed reduced numbers of cells harboring this chromosome (Iezzoni et al., 1997). Patient #3 was a 15-year old phenotypic female with a 46,XY karyotype. She developed a tumor mass on the left and a streak gonad on the right (Hussong et al., 1997).

Two testicular seminoma specimens were obtained from archival samples at the Anatomic Pathology Section, VA Medical Center, San Francisco. At the time of orchiectomy, Patient #1 was a 49-year old male with a tumor mass confined only to the left testicle. Pathological examination revealed a classical seminoma. Patient #2 was a 49-year old male with an advanced and mixed germ cell tumor composed of seminoma, embryonal carcinoma and yolk sac tumor.

Generation of a specific antibody against TSPY

The entire open reading frame of the human TSPY cDNA (Zhang et al., 1992) was subcloned in-frame in the *Eco*R1 site of the expression vector, pAR(ΔR1) (Blanar and Rutter, 1992), a derivative of the pET3a vector. Recombinant TSPY protein was synthesized in bacterial host, BL21DE3 (pLysS) and purified by preparative SDS-PAGE from total lysates of induced bacterial culture. A polyclonal antiserum was generated by repeat immunizations of a New Zealand white rabbit using the service of a commercial vendor (Vancouver Biotechnology, Vancouver, Canada). The specificity of the antiserum was initially assayed by Western blotting against recombinant TSPY protein. The specificity of this antibody to TSPY was further confirmed by both Western blotting and immunocytochemical staining of HeLa cells expressing at high levels a transfected human TSPY gene (Lau, unpublished observations). A polyclonal antibody against the proliferative cell nuclear antigen (PCNA) was purchased from Dako Laboratory, Inc. (Carpinteria, CA). A polyclonal antibody against the human cyclin B1 (synthesized with a bacculuvirus vector in insect cells) was a gift from Catherine Takizawa and David Morgan, Department of Physiology, UCSF. Both antibodies had previously been demonstrated to be specific for the respective antigens in Western blotting and immunostaining studies (Jin et al., 1998; Takizawa et al., 1999; Kömüves et al., 1999).

Immunohistochemistry of tumor tissue sections

Five-micron sections were obtained from archival materials according to established procedure. Immunohistochemical staining was performed as previously described (Kömüves et al., 1999). Heat-induced antigen retrieval pretreatment was utilized in procedures with PCNA and cyclin B1 antibodies. Immunostaining was conducted in a Tris buffer, pH 7.6, containing 4% of bovine serum albumin, 1% gelatin, 0.1% Tween 20, and 500 mM NaCl. The primary antisera were used at 1:500 to 1:1000 dilution ratios. The binding of the primary antibody was detected by reaction with affinity-purified biotinylated goat anti-rabbit IgG, and visualized by either ABC-peroxidase or ABC-alkaline phosphatase reagents. Commercial substrate kits (Vector Laboratories, Burlingame, CA) were used for the enzymatic detections. For the brown and brick-red signals, the DAB and VECTOR NovaRED kits were used respectively with the ABC-peroxidase reagents. For the red signal, the VECTOR RED kit was used with the ABC-alkaline phosphatase reagents. All signals were dependent on the bindings of the respective primary antibodies and were independent of the substrate kits used. After the immunostaining, the sections were counter-stained with hematoxylin. Omitting the primary antibody in the procedure resulted in no signals. Preabsorption of the TSPY antiserum with excess recombinant TSPY protein abolished the staining. The sections were examined and recorded with a Zeiss Axiophot microscope.

Results

Preferential expression of TSPY in tumor germ cells of gonadoblastoma

Histological analysis of the gonadoblastoma specimens from all three patients showed characteristic aggregates of primordial germ cells and sex chord elements. Immunostaining of tissue sections of all four tumors (two patients with unilateral and one patient with bilateral tumors) showed positive staining of cancerous germ cells within these tumor aggregates of all three patients (e.g. Fig. 1A). In particular, the tumor of patient #3 was organized in smaller aggregates with less sex chord elements (Fig. 1B, C), partially resembling the morphology of some forms of germ cell tumors in the testis (e.g. Fig. 1L, M).

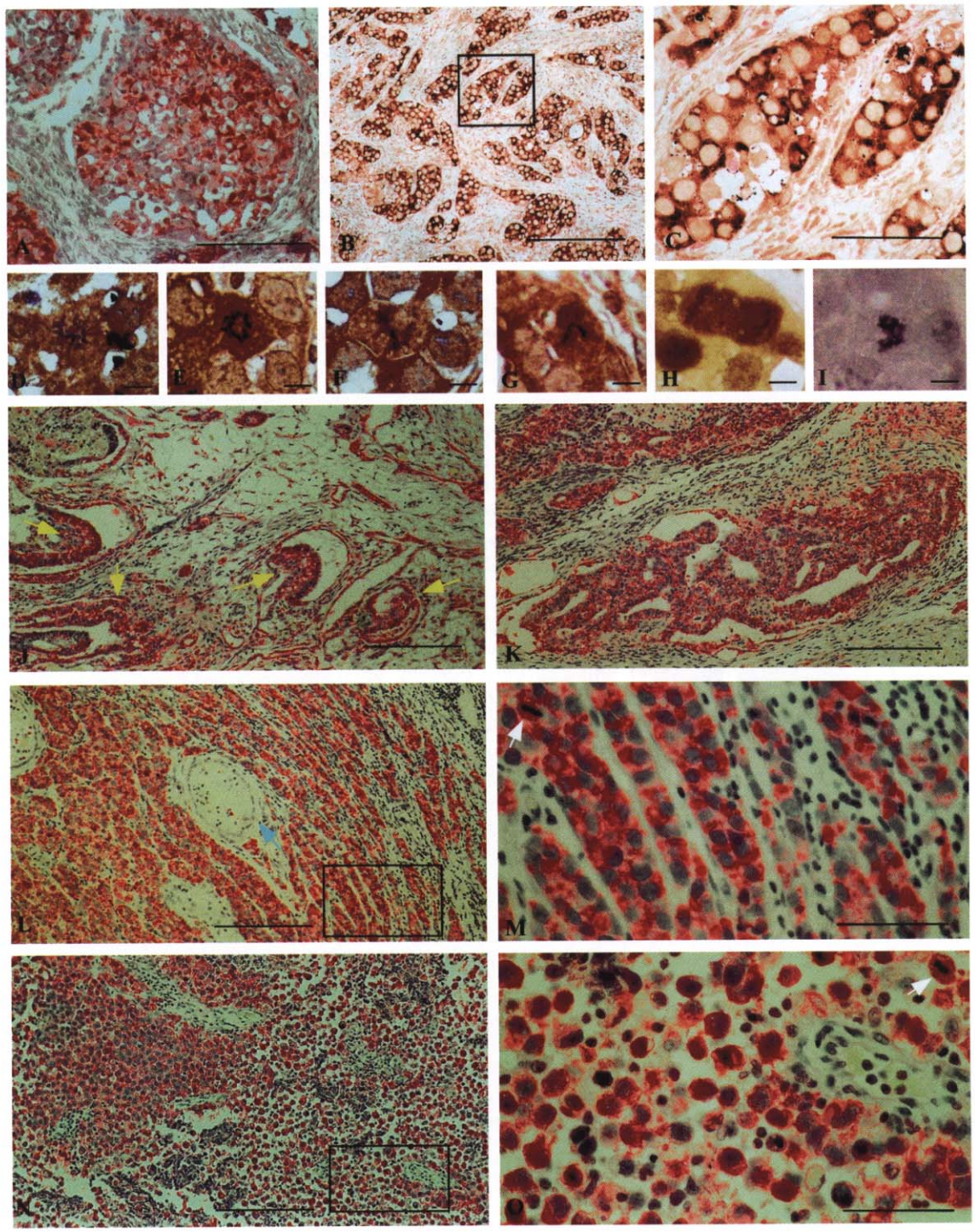

Fig. 1. Immunohistochemical localization of TSPY in gonadoblastoma and testicular seminoma. (**A**) TSPY was primarily located in the germ cells of tumor aggregates of all three gonadoblastoma patients. This figure illustrates an example of immunostaining on sections from Patient #1. (**B**) Patient #3 harbored a gonadoblastoma with less organized aggregates. (**C**) An enlargement of boxed area in **B**, showing prominent cytoplasmic locations of the TSPY protein. (**D–I**) Examples of mitotic cells within the gonadoblastoma from patient #3. Cells in **D–G** were stained with TSPY antibody; **H** with PCNA antibody and **I** a control without primary antibody reaction. (**J–O**) Immunostaining of TSPY on tumor sections from seminoma at early (**J**), intermediate (**K**) and late (**L–O**) stages of the testicular cancer. Yellow arrows in **J** point to possible localized tumor growth areas. Blue arrow in **L** indicates a tubule being abandoned by the invasive growth of the tumor (boxed area enlarged in **M**). **N** Shows an advanced tumor area consisting of large mass of tumor cells and highly undifferentiated and loosely associated embryonal cells (boxed area enlarged in **O**). White arrows in **M** and **O** point to mitotic tumor cells. Positive signals are brick red in **A**; chocolate in **B** and **C**; brown in **D–H**; and red in **J–O**. Bars indicate 50 μm in **A**, **C**, **M** and **O**; 200 μm in **B**, **J**, **L**, and **N**; and 10 μm in **D–I**.

The TSPY protein was prominently localized primarily in the cytoplasm of these large cells (Fig. 1C). In all cases, the sex cord elements and the stroma showed very little reactive signals (Fig. 1A, B). Mitotic cells were readily observed on specimens from this patient (Fig. 1D–I). Omission of the primary antibody or pre-absorption of the antiserum with excess recombinant TSPY protein abolished or greatly reduced the reactive staining of the germ cells in these procedures (e.g. Fig. 1I). Analysis of parallel sections with PCNA and cyclin B1 antibodies showed similar staining patterns as those with the TSPY antibody (data not shown). The signals were primarily located on the nuclei for PCNA while those for cyclin B1 seemed to be associated with both nuclei and cytoplasm of the germ cells. The general staining patterns indicated that TSPY, PCNA and cyclin B1 were co-expressed in the same tumor cells.

TSPY expression in the tumor cells at various stages of testicular seminoma

It has been argued that gonadoblastoma is a precursor form of more aggressive germ cell tumors (Skakkebaek et al., 1987; Jorgensen et al., 1997; Looijenga and Oosterhuis, 1999; Herbst et al., 1999). If TSPY is indeed the candidate for GBY, it would potentially participate in the oncogenic process of other germ cell tumors. Previously, Schneiders and colleagues (1996) had indeed demonstrated an up-regulation of TSPY expression in in-situ carcinoma of the testis, a presumed precursor of germ cell tumors or seminoma. To address the question of whether TSPY may also play a role in more advanced testicular cancer, we have extended our study to include two cases of seminoma using the same immunohistochemical staining technique and specific antibodies against TSPY, PCNA and cyclin B1. They, together, showed various morphological forms potentially representing the different oncogenic stages of these tumors. At the early stages, spermatogenesis might have ceased, thereby depleting normal meiotic cells within the seminiferous tubules. In-situ carcinoma could also occur as a precursor during this initial carcinogenic period (Schnieders et al., 1996). The germ cell-depleted and epithelium-like tubules consisted of a single-layer of cells that might develop into localized multi-layer tumors at various peripheral segments (Fig. 1J, yellow arrows). This transformation progressed until most of the epithelia were lined with multiple layers of tumor germ cells (Fig. 1K). Invasive aggregates of cancerous germ cells eventually evolved from such tubular tumors, abandoning the original tubules (Fig. 1L, blue arrow). In the advanced stages, these aggregates could form a large tumor mass covering a sizable portion of the testis (Fig. 1L, N). Occasionally highly undifferentiated and loosely associated embryonal cells were observed in the late stages of these tumors (Fig. 1O). Presumably, these single tumor cells might have acquired some metastatic properties/potential. Significant expression of the TSPY protein was detected in the tumor germ cells at all stages of these testicular cancers (Fig. 1J–O). Similar to the expression pattern in gonadoblastoma, TSPY was primarily located on the cytoplasm of the tumor cells (Fig. 1M, O). Occasionally, nuclear locations of TSPY were also detected in a few cells. Under each microscopic view, a significant number of mitotic cells could easily be identified (white arrows, Fig. 1M, O). Similar to those in gonadoblastoma, most mitotic cells were stained positively with the TSPY antibody. TSPY expression was at a reduced or insignificant level in the interstitial regions of these testicular cancers.

Immunohistochemical staining of parallel tissue sections with PCNA and cyclin B1 antisera demonstrated similar expression patterns as that of TSPY for both antigens in testicular seminoma (data not shown). Again, PCNA showed a mostly nuclear location while cyclin B1 showed a nuclear and cytoplasmic staining pattern. These observations suggested a possible co-expression of these three proteins in the same tumor germ cells.

Discussion

The mapping of the GBY locus within a small region of the human Y chromosome suggests the existence of a proto-oncogene on this chromosome that predisposes the dysgenetic gonads of XY females to malignancy (Page, 1987; Salo et al., 1995; Tsuchiya et al., 1995). The identification of candidate genes for GBY will not only provide information on the molecular etiology of gonadoblastoma but will also shed light on the contribution of this chromosome to the carcinogenic processes of other male-specific cancers, such as testicular seminoma, germ cell tumors and prostate cancer (Looijenga and Oosterhuis, 1999; Lau, 1999; Lau and Zhang, 2000). Recent studies on TSPY have positioned it to be the most viable candidate for GBY within the critical region on the Y chromosome. First, it is present in the DNAs of gonadoblastoma patients (Salo et al., 1995; Tsuchiya et al., 1995). Its transcripts had been detected by RT-PCR technique in the corresponding tumor tissues (Tsuchiya et al., 1995) and recently by immunostaining in a single case of gonadoblastoma (Hildenbrand et al., 1999). Second, immunostaining studies had demonstrated its expression in spermatogonia in normal testis (Schnieders et al., 1996), suggesting that TSPY may serve a normal function of directing the spermatogonial cells to enter meiosis, a condition for the GBY gene(s) initially postulated by David Page (1987). Third, the TSPY protein is highly homologous to a family of cyclin B binding proteins, including NAP-1 and SET oncoprotein (Tsuchiya et al., 1995; Schnieders et al., 1996), suggesting that it may bind to this mitotic cyclin and be involved in cell cycle regulation and/or cell proliferation (Shin et al., 1999). Results from the present study demonstrate that TSPY is preferentially expressed in the proliferating germ cells within the tumor aggregates in all four samples from three gonadoblastoma patients, further supporting the candidacy of TSPY for GBY.

Although TSPY protein has previously been detected in some forms of testicular tumors, including in-situ carcinoma (Schnieders et al., 1996), our study on the seminoma specimens has clearly demonstrated the high levels of expression of this GBY candidate gene in all stages of these advanced germ cell tumors. Hence, these results, together with those observed by others (Schnieders et al., 1996), establish a direct relationship between the aberrant TSPY expression and the oncogenic process of testicular cancer. Numerous studies have demonstrated that cyclin B binding proteins, such as SET, are either involved in oncogenesis of acute leukemia, Wilm's tumor or modulation

of cell proliferation (von Lindern et al., 1992; Adachi et al., 1994; Carlson et al., 1998; Shin et al., 1999). Although the interactions between TSPY and cyclin B have yet to be demonstrated experimentally, the co-expression of these two molecules on the same tumor cells has raised the possibility that they may indeed interact in vivo. The present study has provided evidence supporting the hypothesis that aberrant expression of TSPY may lead to abnormal cell proliferation and tumor formation (Lau, 1999). Hence, TSPY is not only a key candidate for GBY but may also contribute to the oncogenesis of testicular seminoma.

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Note added in proof

Recently Stuppia et al. (2000) have demonstrated by cloning and sequence analysis that the PRY gene spans 25 kb in size and contains 5 exons. The functional copies of PRY are located in interval 6 on Yq while those on Yp retain only exon 1 and 2 and are likely non-functional.

The Prostate 9999:1–16 (2005)

Unopposed c-MYC Expression in Benign Prostatic Epithelium Causes a Cancer Phenotype

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BACKGROUND. We have sought to develop a new in vivo model of prostate carcinogenesis using human prostatic epithelial cell cultures. Human prostate cancers frequently display DNA amplification in the 8q24 amplicon, which leads to an increase in the copy number of the c-MYC gene, a finding that suggests a role for c-MYC in human prostate carcinogenesis. In addition overexpression of c-MYC in transgenic mouse models results in prostatic carcinogenesis.
METHODS. We took advantage of the ability of retroviruses to integrate foreign DNA into human prostatic epithelium (huPrE) to generate cell lines that overexpress the c-MYC protooncogene. These cells were recombined with inductive rat urogenital sinus mesenchyme and grafted beneath the renal capsule of immunocompromised rodent hosts.
RESULTS. The resultant tissue displayed a phenotype consistent with a poorly differentiated human prostatic adenocarcinoma. The tumors were rapidly growing with a high proliferative index. The neoplastic cells in the tumor expressed both androgen receptors (AR) and prostate-specific antigen (PSA), both characteristic markers of human prostate cancers. Microarray analysis of human prostatic epithelial cells overexpression c-MYC identified a large number of differentially expressed genes some of which have been suggested to characterize a subset of human cancers that have myc overexpression. Specific examples were confirmed by Western blot analysis and include upregulation of c-Myb and decreased expression of PTEN. Control grafts using either uninfected huPrE or using huPrE cells infected using an empty vector expressing a green fluorescent protein tag gave rise to well differentiated benign prostatic glandular ducts.
CONCLUSIONS. By using a retroviral infection strategy followed by tissue recombination we have created a model of human prostate cancer that demonstrates that the c-MYC gene is sufficient to induce carcinogenesis. © 2004 Wiley-Liss, Inc.

KEY WORDS: **myc; tissue recombination; retroviral gene transfer; prostate cancer**

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INTRODUCTION

Prostate cancer is the single most diagnosed cancer in men and a major cause of mortality/morbidity within North America and Europe [1–3]. The introduction of routine PSA testing has resulted in earlier detection of prostate cancer and appears to be resulting in a decrease in disease-specific death rates [4]. While certain proteins such as TSPY have been found to display altered expression in very early cancer [5], there has not been sufficient characterization of PCa to identify many potential progression pathways that characterize prostate cancer [6]. Therefore, unlike the well-characterized pathway of acquired mutations displayed by colon cancer, there is not a clearly defined pathway for the progression and development of malignant disease in the prostate.

c-MYC is a transcription factor that belongs to the myc/mad/max family of Basic-helix-loop-helix-zipper (bHLHZ) proteins. Three closely related members make up the MYC family (c-MYC, L-MYC, N-MYC) and although they have very distinct patterns of expression, evidence exists that the proteins are able to compensate, to some extent, for the loss of one family member [7]. However, both c-MYC and N-myc knockout mice exhibit embryonic lethality [8,9].

Myc forms a heterodimeric transcription factor complex with its partner Max [10]. In this state Myc/Max is capable of binding to its DNA recognition site, the so-called E-box [core sequence (CACGTG)]. Max/Mad heterodimers also bind the E-box and act as transcriptional repressors presumably repressing genes induced by Myc/Max [11]. Like myc, both Max and Mad have related family members, capable of modulating this pattern of induction/repression by binding to each other and modulating the availability of the E-box. Myc also appears to be capable of binding and sequestering several other regulatory factors such as Sp-1 and Miz-1, causing transcriptional modulatory effects not associated with E-box binding [12–14].

Cell proliferation, differentiation, and apoptosis are all responses regulated by myc expression. The Myc protein acts as a cell activator that relies on other accessory proteins to specify the nature of the response. The proliferation pathway is mediated by Myc's ability to activate several cyclins, including cyclin E [15] and cyclin D2 [16,17]. The activation of cyclin D2 causes sequestering of $p27^{kip}$ from cyclin E and driving the cell into S phase. Myc also indirectly reduces expression of $p21^{WAF1}$ and $p15^{ink4b}$ [18,19], both of which are involved in cell cycle arrest.

Myc overexpression/deregulation has been implicated in numerous neoplastic transformations both in human disease and transgenic mouse models [20–28]. Furthermore, inactivation of the myc gene has been shown to elicit regression of Myc-induced tumors in the absence of novel mutations [23,29,30].

c-MYC was the first oncogene to be recognized as being overexpressed in human prostate cancer [28]. However, the precise role played by c-MYC in human prostate cancer is unclear in part due to the amplification of the 8q24 amplicon. This amplicon is particularly rich in genes, several of which [e.g., c-MYC [31], NOV [nephroblastoma overexpressed gene], EIF3S3 [eukaryotic translation initiation factor 3 subunit 3], HAS2 [hyaluronan synthase2] [32], KIAA0196 [33], and PSCA [34]] are expressed in prostate and have either oncogenic or tumor suppressor potential. FISH analysis has identified amplification of the 8q24 amplicon [32,35–37] in a large percentage of human adenocarcinomas [38,39] and some prostate intraepithelial neoplasias (PIN) [37].

Mouse models of prostatic neoplasia have been generated in which the c-MYC gene was expressed from either the probasin promoter [20] or C(3)1 promoter [22]. Two probasin promoter variants were used in one study; these promoters share the same prostate specificity but differ in promoter activity. Both the Hi-Myc (ARR_2PB-myc) and Low-Myc (sPB-myc) animals develop mouse PIN (mPIN) and invasive carcinoma but the time to development and progression differs by approximately 6 months [20]. While these probasin-Myc transgenic mice apparently progress from mPIN to prostatic adenocarcinoma the relationship of PIN to adenocarcinoma in humans remains unsubstantiated. The C(3)1 promoter is a weaker promoter than the probasin constructs. Mice carrying the C(3)1-Myc transgene fail to develop adenocarcinoma within their lifetime although they do develop mPIN-like lesions [22]. These data suggest that a low level of myc expression correlates with mPIN development while the progression to adenocarcinoma requires elevated c-MYC levels.

Given that c-MYC is reported to be overexpressed and the gene amplified in human prostate cancer, and because transgenic mouse models overexpressing c-MYC in the prostate have a dose related progression towards malignancy, we decided to test the ability of cMYC to transform human prostatic epithelium. To do so in vivo a tissue recombination model was used to follow prostatic carcinogenesis in response to overexpression of c-MYC.

MATERIALS AND METHODS

Human Cell Culture

Human prostate tissue samples were obtained from the Vanderbilt Tissue Acquisition Core via the Department of Pathology in accordance with Vanderbilt IRB protocols. Cores 6 mm in diameter were removed from

radical prostatectomy samples and were sampled by histologic analysis on frozen sections to determine the nature (benign vs. malignant vs. severe inflammation) of the tissue contained within the core.

Benign tissue was cut into 2 mm cubes using sterile scalpels. After washing in RPMI (Gibco, Carlsband, CA) 5% FCS (Atlanta Bioscience Atlanta, GA) the tissue was plated on Primaria™ tissue culture flasks with sufficient medium to wet the plate and create a strong surface tension (1.2 ml/25 cm^2). After cell attachment had taken place (~12 hr) the volume of medium was increased. Tissue obtained from tissue recombination grafts was reintroduced into culture in an identical manner.

Tissue Culture Medium

Tissue culture medium for human prostatic epithelial cells (huEpi mix) consisted of: [RPMI 1640, 1% ITS (Insulin Transferrin Selenium), 1% Antibiotic/Antimycotic (all from Gibco)], 2.5% charcoal stripped serum (Atlanta Bioscience), BPE (Bovine Pituitary Extract) 1:250 (Hammond Cell Tech, Winsor CA), Cholera toxin (1 μg/ml), and Epidermal Growth Factor (0.01 μg/ml) (Sigma, St. Louis, MO).

LZRS Retroviral Plasmid Construct

The plasmid LZRS c-MYC/EGFP (Fig. 1) was constructed utilizing the LZRS-EGFP backbone (Nolan Laboratory, Stanford, CA). The CMV promoter was excised from pIRES-EGFP (Clontech, Palo Alto, CA) as a BglII/BamHI fragment. The fragment was ligated into the BamHI site of the LZRS-EGFP backbone to give C7Δ.

The human c-MYC cDNA clone (BC000917) was obtained from ATCC (Rockville, MD) and amplified by PCR using a 5′ primer specific to translational start site and a 3′ primer containing an XhoI restriction site and the consensus sequence for the translational stop site with subsequent deletion of any polyadenylation sites. After PER amplification, the product was gel purified, and cloned into poem T-Easy (ProtÕgÕ, Madison, WI). Following DNA sequence verification of the cloned product the c-MYC coding region was excised using EcoRI/XhoI and sub cloned into the EcoRI/XhoI sites of pLZRS-EGFP to give C7-Myc.

Viral Production

Amphotrophic ϕNXA packaging cells were obtained from ATCC [under an MTA from the Nolan laboratory Stanford (www.stanford.edu/group/nolan)]. These cells were maintained in 5% FCS/RPMI 1,640 with antibiotic surveillance. The ϕNX packaging cell lines were reselected with both hygromycin B and diphtheria toxin (Sigma) every 3–4 months. The LZRS retroviral constructs were transfected into ϕNX cells that were 70%–80% confluent. Routine transfection took place using 25 cm^2 flasks. Lipofectamine 2000 (Invitrogen, Grand Island, NY) was optimally used at a

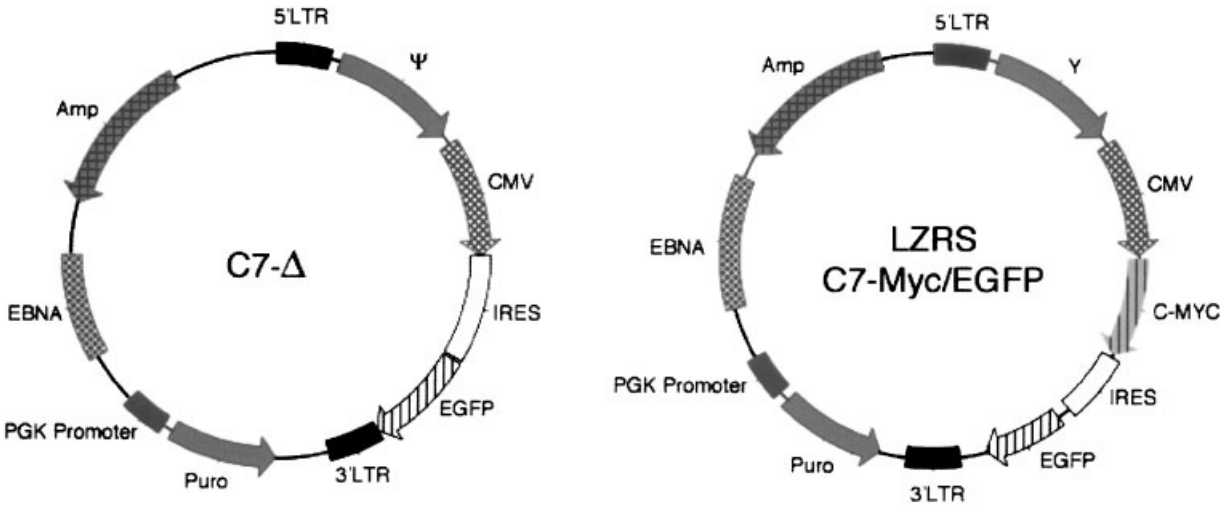

Fig. 1. Retroviral constructs, control vector C7Δ and LZRS C7-MYC/EGFP. pLZRS is a retroviral vector derived from the Moloney murine Leukemia virus (MoMuLV). The gene of interest (c-MYC) and the Enhanced Green Fluorescent Protein (EGFP) are expressed from a bicistronic message under the control of the cmv promoter. The 5′ viral LTR controls expression of the transcript that contains Ψ (the extended viral packaging signal). The retroviral particle contains, and integrates into the genome, only the genetic information between and including the 5′ and 3′ LTRs'. pLZRS does not contain the structural genes necessary for viral formation and replication, these are provided by the packaging cell line ϕNX. Other components of pLZRS such as the puromycin resistance gene and EBNA (EBV episomal functions) contribute to stability and selection of the plasmid in the packaging cell line. pLZRS also includes the pUC origin of replication and E. coli Amp gene for propagation and antibiotic selection in bacteria.

final concentration of 3 μl/ml according to manufacture's protocols. Eight hours post transfection the ϕNX cells were given fresh RPMI/5% FCS and incubated overnight at 37°C. Viral supernatant was removed in the morning and the cells split if necessary and moved to a 32°C incubator to ensure greater viral stability. The viral supernatant was spun at 3,000 rpm and passed through a 45 μm filter to ensure the absence of contaminating ϕNX cells. The viral supernatant was then supplemented with 4 μg/ml Polybrene (hexadimethrine bromide, Sigma) and either stored at −70°C or used immediately.

Viral Infection

The viral supernatant containing the c-MYC/EGFP or C7-delta control retrovirus was diluted 1:1 with huEpi mix that was double strength with respect to the added constituents and the polybrene concentration was corrected to 4 μg/ml. The viral medium was then placed on the huPrE cells (three patients from which both TZ and PZ cores were put into independent culture dishes in duplicate) in culture and replaced 8 hr later. Primary cells were incubated at 37°C as their rate of cell division was severely compromised at 32°C, the increased stability of the viral particles at 32°C therefore could not be utilized when working with huPrE but proved useful when certain immortal cell lines were infected, data not shown. Successive rounds of infection over 5 days were employed to generate infected cells. Owing to the growth patterns of the huPrE we found that daily infection would ensure the presence of virus during the initial outgrowth of cells and therefore generate the maximum number of infected cells. Infection rates were monitored using fluorescence microscopy of the bicistronic EGFP tag. After infection the cells were maintained in culture until use. The cells were examined by a clinical pathologist to assess any phenotypic differences in the infected cells.

Culture of Infected Cells

Following infection, a proportion of the cells were used as a source of epithelium in tissue recombinants. The remaining cells were maintained in culture for a further 16 passages with batches frozen in liquid nitrogen at different points. All remaining cells were frozen after passage 16. Cells were examined for the continued expression of the EGFP tag using fluorescence microscopy; expression of AR, PSA, and c-MYC was monitored using Western blot analysis.

Tissue Recombination

Tissue recombinants were prepared, as previously described [40,41]. Briefly, pregnant rats were obtained from Harlan (Harlan, Indianapolis). Rat urogenital sinus mesenchyme (UGM) was prepared from 18-days embryonic fetuses (plug date denoted as day 0). Urogenital sinuses were dissected from fetuses and separated into epithelial and mesenchymal components by tryptic digestion, as previously described. UGM was then further reduced to single cells by a 90 min digestion at 37°C with 187 U/ml collagenase (Gibco). Following digestion, the cells were washed extensively with RPMI-1640 tissue culture medium. Viable cells were then counted using a hemacytometer, with viability determined by trypan blue exclusion. Epithelial cells were released from tissue culture plates using trypsin. Trypsin was neutralized and the cells washed and counted using a hemacytometer. Cell recombinants were prepared by mixing 100,000 epithelial cells with 300,000 stromal cells. Cells were pelleted and resuspended in 50 μl of neutralized type 1 rat tail collagen prepared as, previously described [42]. The recombinants were allowed to gel at 37°C for 15 min and were then covered with growth medium and cultured overnight. They were then grafted beneath the renal capsule of adult male severe combined immunodeficient (SCID) mice [C.B.17/IcrHsd-scid mice (Harlan)].

Subcutaneous Grafting

Epithelial cells (100,000) were pelleted and resuspended in 50 μl of neutralized type 1 rat tail collagen prepared, as described previously [42] and placed under the skin of adult male SCID mice [C.B.17/IcrHsd-scid mice (Harlan)]. Some of these subcutaneus grafts were surrounded in matrigel (BD Biosciences, Bedford, MA) at the time of grafting.

Tissue Recovery, Fixation, and Processing

Mice were sacrificed by Isoflurane inhalation followed by cervical dislocation according to Vanderbilt animal care protocols. The kidney and attached graft together with internal organs were removed and examined at the gross level. The c-MYC grafts owing to their substantial size were divided into several pieces. Those fragments containing the kidney were fixed in 10% neutral buffered formalin, as were the internal organs of the host. The remaining tissue was then divided to give representative portions for (1) RNA extraction, (2) Protein extraction, and (3) for further tissue culture. Tissue destined for RNA extraction was cut into small pieces and immersed in RNA LATER (Ambion, Austin, TX) according to manufacturer's instructions. Tumor tissue for protein extraction was snap frozen on dry ice and stored at −70°C until required. The third portion of the tumor was removed

for tissue culture or frozen [43] to allow extraction of viable cells.

Culture of Tumor-Derived Epithelial Cells

Tumor tissue was minced and placed in culture in a minimal volume of tissue culture medium (as per primary culture). Cells were passaged using trypsin and then frozen. Continued transduced gene expression in these cells was confirmed by fluorescence microscopy to detect the expression of EGFP and by Western blotting to confirm continued expression of c-MYC.

Antibodies

For immunolocalization studies the following antisera were used. Androgen receptors (AR) were detected using a rabbit polyclonal antibody (*sc*-816) raised against a peptide within the N-terminal domain of hAR, EGFP was detected using a mouse monoclonal to the full length GFP that detects all GFP variants (*sc*-9996). c-Myb (*sc*-8412), PTEN (*sc*-7974*sc*-9996), p63 (*sc*-8343) (Santa Cruz Biotechnology, Santa Cruz, CA). c-MYC was detected using a mouse monoclonal (NCL-c-MYC) raised against full-length recombinant human protein obtained from Novocastra (Burlingam, CA). Ki67 (M-7240), PSA (A-0562), broad-spectrum Keratin antibodies (Z-0622) were obtained from DAKO (Carpinteria, CA), and keratin 8, 14, and 18 [clones LE41, LL001, and LE61 gifts from Prof. E.B.Lane, Dundee University, UK [44,45]].

Protein Extraction

Tissue extracts of human prostate epithelial cells maintained in tissue culture for one passage, and c-MYC infected huPrE cells were prepared by homogenization in 400 μl cold buffer A (10 mM HEPES pH 7.9; 10 mM KCl: 0.1 mM EDTA; 0.1 mM EGTA; 0.1 mM DTT; 1× protease complete (Roche, Indianapolis, IN). The cells were allowed to swell on ice for 15 min, after which 25 μl of a 10% solution of Nonidet NP-40 (Sigma) was added and the tube vortexed vigorously for 10 sec. The homogenate was then centrifuged for 30 sec in a Microfuge. The supernatant was snap frozen and stored at −70°C.

Western Blotting Analysis

Tissue extracts from huPrE epithelial cells grown in culture and their c-MYC infected counterparts as well as extracts from the BPH1 prostatic epithelial cell line were run on denaturing mini gels containing an acrylamide gradient from 4%–20% (w/v) polyacrylamide (Invitrogen). Gels were run in MOPS/SDS running buffer (50 mM 3-[*N*-morpholino] propane sulfonic acid [MOPS], 50 mM Tris base, 0.1% SDS, 1.025 mM EDTA [pH 7.7] for 35 min at 200 mA. Samples were blotted onto PVDF membrane (Invitrogen) using transfer buffer (25 mM bicine, 25 mM Bis-Tris, 1.025 mM EDTA, 50 nM chorobutanol [pH 7.2] (Invitrogen) in the mini gel tank according to the manufacturers instructions. Thereafter, membranes were blocked for 2–3 hr at room temperature in BLOTTO (5% nonfat dried milk powder [Difco]) dissolved in Phosphate buffered saline (Sigma) containing 0.1% Tween-20 (PBST). Membranes were incubated overnight in Blotto with the any one of the antibodies (anti c-MYC 1:800, AR 1:1,000, c-Myb 1:600, GFP 1:2,000, E-Cad 1:1,000, PTEN 1:800). Bound antibodies were detected using appropriate secondary antibodies (1:4,000 peroxidase conjugated Donkey anti rabbit/Sheep anti mouse [Amersham Pharmacia Biotech, Piscataway, NJ] and 1:2,000 rabbit anti goat [Santa Cruz]) and the enhanced chemiluminescence visualization system (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

Immunohistochemistry

Deparaffinized, slide mounted sections were rehydrated and then subjected to heat induced antigen retrieval using the commercial retrieval buffer (H-3300) from Vector Laboratories, Brlingham, CA. The sections were microwaved for 15 min at a power that ensured continuous but not excessive boiling. Slides were permitted to cool to room temperature prior to incubation with 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase. After washing in PBS the slides were blocked in Clean Vision™ from ImmunoVision Technologies for 15 min. This blocking optimized the use of monoclonal antibodies on tissue recombinants in which an immunocompromised mouse host was the graft host. The antibodies towards GFP (Santa Cruz,), Ki67, PSA, and broad spectrum Keratin (DAKO) were all used at 1:200 (p63 used at 1:1,000) [diluted in 1:4 normal swine serum (broad spectrum Keratin, PSA, p63) or normal rabbit serum (GFP, Ki67, keratins 8, 14, 18) in PBS/5% BSA]. All antibodies were incubated on sections overnight at 4°C. Sections were then incubated with the appropriate biotinylated secondary antibodies for 1 hr. [Broad spectrum Keratin, p63 and PSA, swine anti rabbit (DAKO), and for GFP, keratin 8, 14, 18 and Ki67 rabbit anti mouse, (DAKO), both of which were diluted 1:500 in the appropriate normal serum (see above)]. After appropriate washing steps the sections were incubated in ABC-HRP complex (Vector) for 30 min and washed extensively. Bound antibodies were then visualized by incubation with 3,3′-diaminobenzidine tetrahydrochloride (liquid DAB, DAKO). Sections were counter-

stained with hematoxylin. Images were captured onto a computer using a Zeiss microscope equipped with an AxioCam camera (Zeiss) and software.

Identification in Histological Sections of Species Origin of Cells in a Tissue Graft

Staining with the Hoechst 33258 dye (Sigma) was performed, as previously described [46]. The Hoechst dye Sections were examined by fluorescence microscopy. Host mouse cells contain several small discrete intranuclear fluorescent bodies, which are absent in cells from either rat or human allowing us to confirm that the tumor is not derived from the host mouse.

RNA Isolation

Tissue was rapidly excised from the outer portions of the graft and placed in 10x v/v RNA LATER (Ambion, Austin, TX). The tissue was then refrigerated prior to use 1–5 days after removal from the host. The tissue was dissected in a Petri dish containing RNA LATER to remove any kidney tissue (none was actually visible in any of the tissues) or obviously necrotic tissue (this tissue was very soft and white, resembling cotton candy).

RNA was isolated using the Qiagen mini RNA Easy kit according to the manufacturers instructions (Qiagen, Valencia, CA). The RNA was DNAse treated again using Qiagen reagents as detailed in the RNA easy protocol. The RNA concentration was then determined spectrophotometrically and the RNA aliquoted and snap frozen at −70°C.

RNA Labeling and Hybridization

All RNA and cDNA manipulations and cDNA array hybridizations were undertaken by technical support staff within the Vanderbilt Microarray Shared Resource (http://array.mc.vanderbilt.edu) using the protocols outlined on their website http://array.mc.vanderbilt.edu/support/protocols.htm. The use of tissue culture cells permitted the use of the standard cDNA labeling without amplification of the RNA. The microarray used for our experiments was the human 11K comprised of the Research Genetics human clone set.

cDNA Array Analysis

Data was normalized by Lowess sub grid and Gene Pix Pro Software was used to analyze differentially expressed genes between the two analysis groups. The groups consisted of huPrE grown in culture and infected with the LZRS-myc virus.

RESULTS

Infection of Hupre With a Retrovirus Containing C-Myc/Egfp Elicits Phenotypic Changes In Vitro

The huPrE cells grown on tissue culture plastic have a characteristic cobblestone appearance (Fig. 2). The presence of high levels of cholera toxin severely suppressed fibroblastic growth resulting in an almost pure epithelial cell population, as determined by visual examination. The rate of emergence of epithelial cell sheets from individual tissue fragments was highly variable both with respect to 'flask to flask' variation-using tissue from a single patient and with respect to

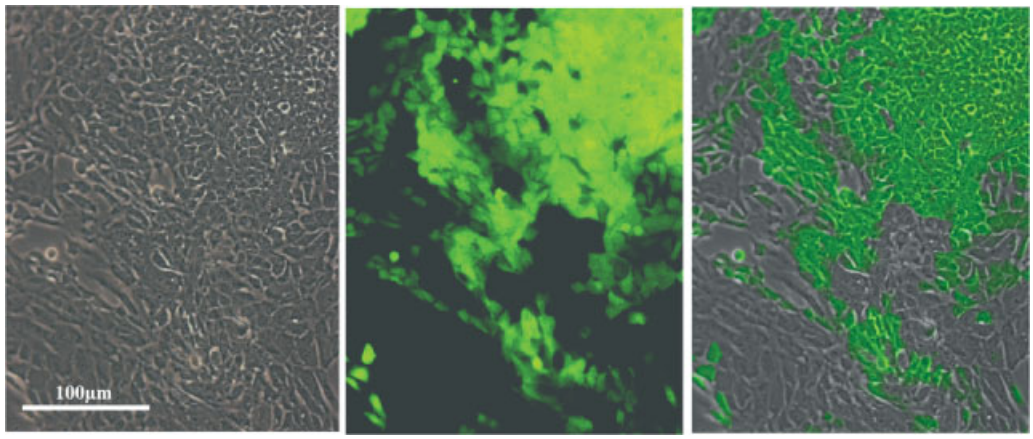

Fig. 2. Phase contrast photomicrograph of human prostate epithelium in cell culture 4 days after infection (Left panel). Note the typical cobblestone morphology in lower left hand side compared to the denser cells present in the upper right hand side. (Middle panel) Fluorescent image of human prostate epithelium culture 4 days post infection. Note that approximately one half of the cultured cells strongly express EGFP due to infection by the C7-Myc retrovirus. (Right panel) overlay, confirming that the small densely packed cells express EGFP while those with the cobblestone morphology do not.

'patient to patient' variation. Infection was started on day 2 in culture regardless of the presence/absence of visible epithelial cell outgrowths. After 2–5 rounds of infection a very large number of cells (20%–40%) on the margins of the growing sheet of epithelial cells expressed EGFP, suggesting an extremely efficient infection. At this point, infection was halted and the cells allowed to proliferate for 72 hr. The cultured cells displayed an altered morphology. Instead of a uniform monolayer of epithelial cells with a cobblestone appearance, there were discreet nests of tightly packed cells. The nests had well defined borders and were surrounded by cells displaying the expected normal phenotype (Fig. 2). When visualized on an inverted fluorescent microscope the nests displayed uniform EGFP fluorescence while the surrounding cells did not express EGFP.

All of the c-MYC/EGFP infected cell cultures observed (three patients from which both TZ and PZ cores were put into independent culture dishes in duplicate, a total of 12 independent cultures) exhibited similar morphological changes. The cells were irregular in shape and markedly smaller, the cytoplasm to nuclear ratio was decreased, and the nucleus was in many cases irregular in shape with prominent nucleoli. The cells also formed multiple layers within the nests, and many non-adherent viable cells were observed. The 'nest' phenomenon was observed until the cultures were trypsinized and split. Subsequent cultures initially displayed both phenotypes of cells but the morphologically normal cells were rapidly lost as the EGFP-expressing epithelial cells rapidly colonized the tissue culture plates. Within 10 days the entire culture consisted of EGFP expressing cells that varied widely in their fluorescence (and by subsequent analysis c-MYC expression). Over time the EGFP expression became more uniform but a range of EGFP expression has always been observed throughout the cultures. This suggests that the cultures represent a variety of clones resulting from multiple initial infections.

HuPrE infected with the LZRS C7Δ (the 'empty vector') exhibited normal cobblestone morphology and typically infect with relatively low efficiency $\leq$1% at low passage number, the control infections are less efficient than infections with the C7-Myc retrovirus. The Green EGFP infected cells were indistinguishable from their uninfected neighbors under phase contrast and could only be identified by EGFP expression. The C7Δ-infected cells could be passaged a maximum of five times before they became senescent.

The C7 Myc infected cells were maintained through 16 passages and showed no signs of senescence during this period. They continued to express both EGFP and c-MYC as well as prostatic epithelial markers including AR and PSA.

Hupre/C-Myc-Egfp Plus RugmTissue Recombinants Form Rapidly Growing Adenocarcinomas Expressing Human Prostatic Markers

Tissue recombinants of huPrE and rUGM were prepared. Control grafts contained either uninfected or C7-Δ-infected epithelium. Recombinants using C7-myc contained different percentages of c-MYC-infected cells (from 10% to 50% dependent on the time post infection that the cells were grafted). All grafts were composed of 100,000 epithelial cells and 300,000 rUGM cells. Host mice carrying myc-expressing grafts were sacrificed after 28 days due to the large size of the graft, which exceeded the size of the normal kidney (Fig. 3). Recombinants composed of C7Δ PrE and rUGM grafted to the contralateral kidneys of experimental hosts were very small and poorly developed at 28 days post grafting. In separate experiments, using a 3-month time point fully differentiated prostatic structures expressing PSA and AR were seen. Control grafts maintained a benign histology throughout (Fig. 3). The phenotype and timing of developmental events in these control grafts is consistent with previously published results using human prostatic epithelial organoids [47].

Initial inspection of the large c-MYC expressing grafts, indicated several very large blood vessels located on the surface of the graft and areas of white necrotic tissue in areas devoid of obvious blood vessels (Fig. 3). The kidney tissue was readily apparent upon subsequent dissection and was essentially normal.

C7-Myc cells grafted subcutaneously into the flank of SCID mice developed large tumors after 6 weeks (data not shown) that were indistinguishable from C7-Myc recombined with rUGM. The slightly longer time frame probably reflects the slower recruitment of blood vessels to a subcutaneous graft site compared to the sub renal graft site.

Thin smears of surface cells from the tumors expressed EGFP when exposed to fluorescent light (data not shown). These cells were placed into tissue culture to confirm EGFP expression and their human origin.

Expression from the CMV promoter is maintained throughout the growth of the graft. We have previously observed promoter downregulation (unpublished data) in cells carrying genes under the CMV promoter introduced via retroviral integration possibly via methylation events consistent with those described in transgenic mice [48,49]. The EGFP levels within the cells placed into reculture maintained a range of EGFP expression consistent with cultures prior to grafting suggesting that such repression had not occurred in these tumors.

In all the huPrE/c-MYC tumors the grafts consisted of cells strongly staining with hematoxylin. The cells characteristically exhibited large irregular nuclei, pro-

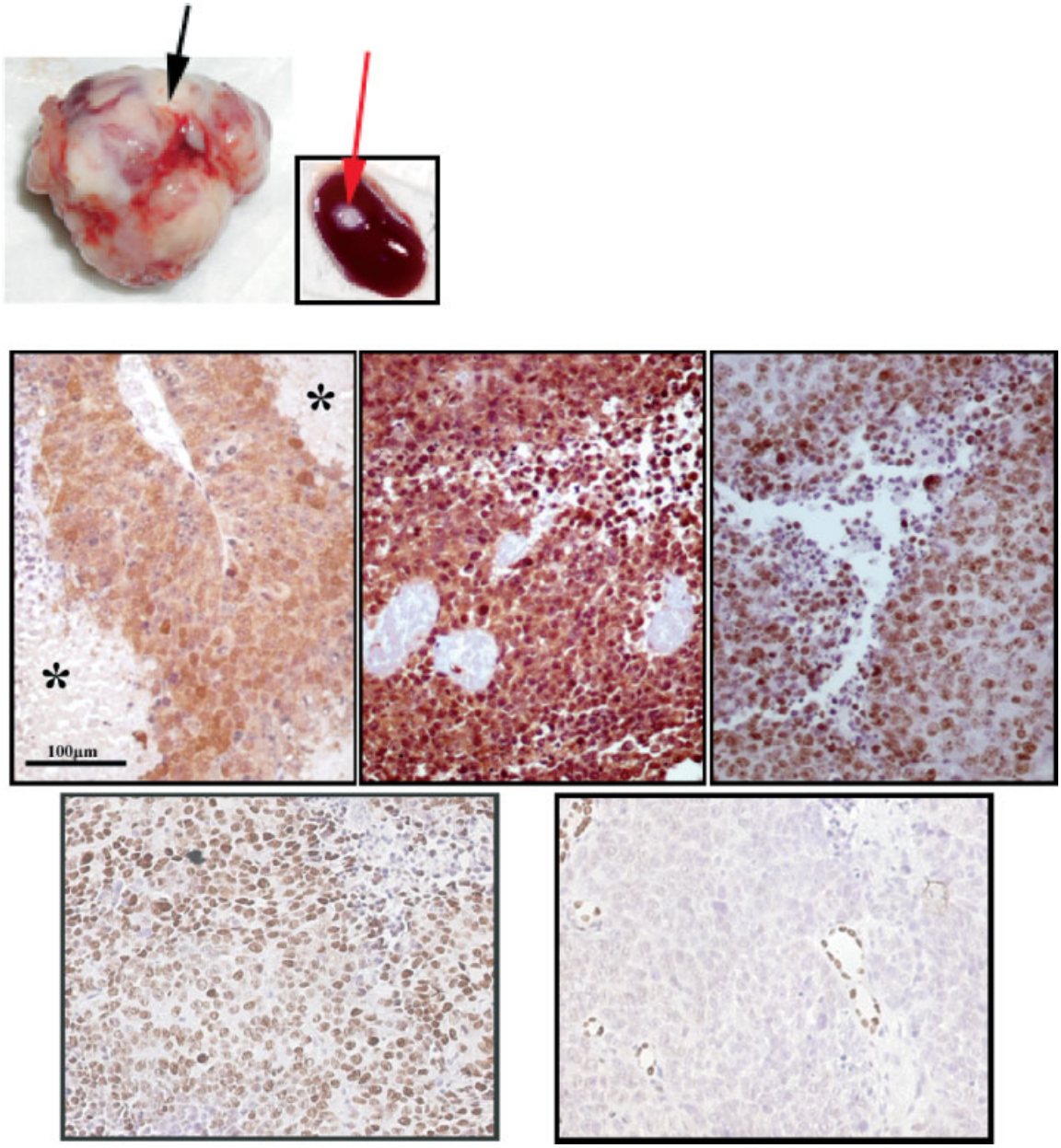

Fig. 3. Gross anatomy of mouse kidney with a c-MYC overexpressing prostate epithelial cell recombinant xenograft (top left). Note the encasement of the kidney by the tumor (black arrow). The control kidney carrying the C7Δ PrE/rUGM graft is displayed on the right, the small xenograft is barely visible (red arrow). Photomicrograph showing the poorly differentiated c-MYC expressing human prostatic tumors (middle/bottom panel) strongly express enhanced green fluorescent protein (middle left) PSA [(center)* indicates necrotic tissue]. The high mitotic rate observed in the tumor was confirmed by a very high index of statining with Ki67 (middle right). Bottom panel (left to right) AR, and p63.

minent nucleoli, and dense cytoplasm. A large number of dividing cells were present in a single field and many of the mitotic figures were clearly abnormal (Fig. 3). No fibromuscular stroma was present in the grafts nor was any apparent at the graft extremities. Irregular areas of necrotic tissues extended throughout the grafts and predominated in the interior, but did not interfere with the kidney. Areas of living tissue surrounded the few blood vessels that populated the graft interior. Immunohistochemical analysis of the C7-myc induced tumors revealed that these retained expression of two key markers of prostate tissue, AR and PSA. In addition the tumors expressed EGFP confirming their origin. Tumors expressed keratins 8 and 18 and lacked p63 and keratin 14 staining suggesting a luminal rather than basal cell origin. This is consistent with the profile seen

in human prostate cancer. The proliferation rate of the cells was extremely high as indicated by the almost universal presence of Ki67 in the nuclei (Fig. 3). This was consistent with the observation of extremely rapid tumor growth.

No infiltration was observed into the kidney, a well dealinated border was observed and the kidney morphology was normal with no obvious compression or damage (Fig. 3). Hoechst 33258 staining confirmed that none of the epithelial cells observed were of mouse origin and that no 'non mouse' cells were present in the kidney (data not shown). Some areas displaying p63 positive cells were observed within the graft, these were determined (by a trained pathologist) to be mouse kidney structures caught in cross section, providing a good internal positive control for the tumor cells, which were universally negative for basal cell markers (Fig. 3).

Molecular Characteristics of Tumor-Derived Cells

A microarray analysis comparison of the cells derived from the C7-myc tumors and of primary epithelial cultures from the same patient revealed a number of changes (Table 1). As might be expected many genes were seen to be regulated in response to c-MYC overexpression. Attention in this analysis was focused upon genes known to be regulated in tumors. Of particular note was the observation that PTEN and E-cadherin expression was seen to be suppressed and c-Myb expression was observed to be upregulated in the tumorigenic cells. This observation was confirmed by Western blotting to examine expression of these proteins (Fig. 5). Western blotting analysis also confirmed the continued overexpression of c-MYC, and the expression of both AR and EGFP.

DISCUSSION

The present study demonstrates that c-MYC overexpression is sufficient to drive normal human prostatic epithelium to a metastatic tumor within a tissue recombination model. Retroviral infection of normal prostate epithelium with the EGFP expressing empty vector resulted in benign prostatic architecture indicating that MYC overexpression not infection/integration

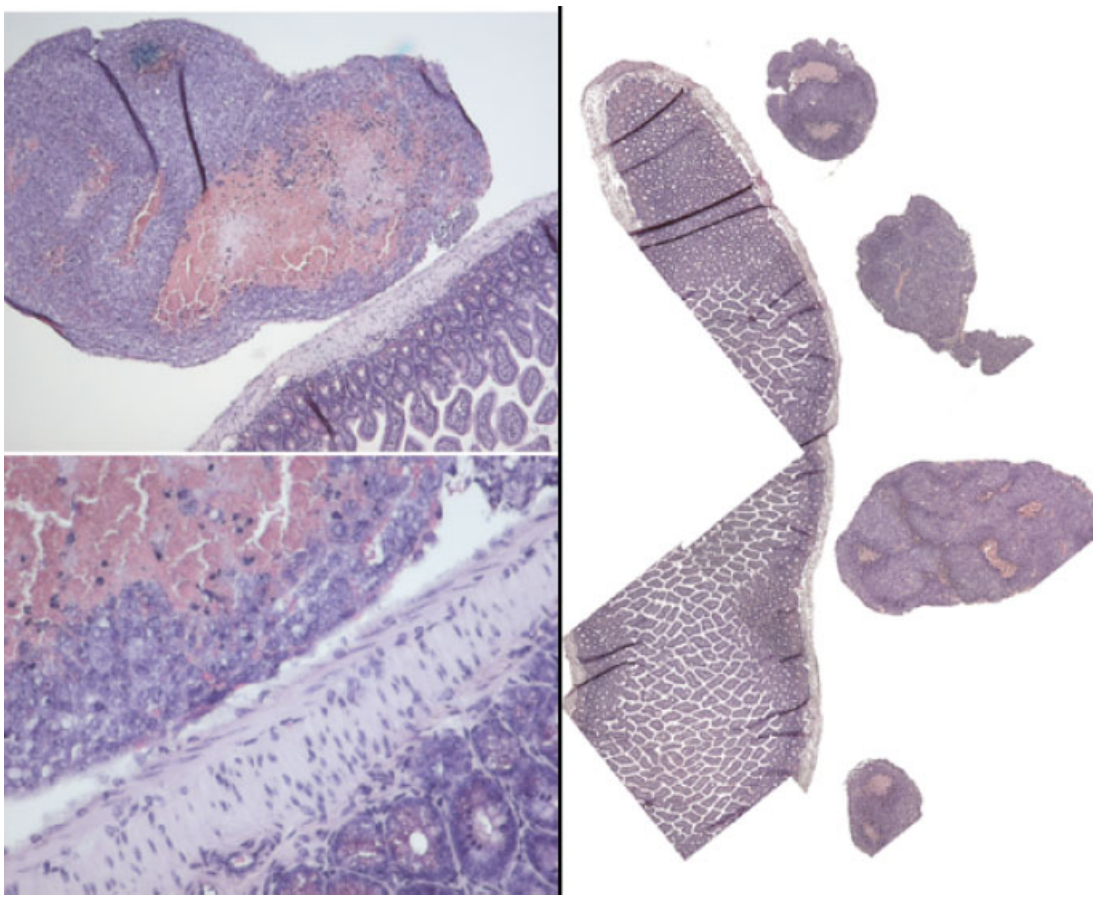

Fig. 4. Histopathologic appearance of metastatic lesions. Multiple metastatic lesions were found in mice as focal growths predominantly following the line of the intestinal tract. H&E high magnification of tumor immediately adjacent to the intestine (left panel top & bottom). Low magnification composite (right panel) showing three metastatic nodules that were attached to the host mouse intestine.

TABLE 1. Microarray Data Set of mRNA Showing 2-fold Differences in Levels Between C7-Myc PrE and C7Δ PrE

Category	Gene	GenBank ID	Description	ACF
Transcription Factors	ARHE	NM_00518	Ras homolog gene	3.67
	MAFG	NM_00239	v-maf oncogene homolog G	3.27
	SON	NM_05818	Similarities with MYC MOS	−2.29
	HRASLS3	NM_00706	HRAS-like suppressor 3	−2.29
	TCF7	NM_00320	Transcription factor 7	
	TCF12	NM_00320	Transcription factor 12	−4.67
	VAV3	NM_00611	vav 3 oncogene	−1.36
	NFE2L2	NM_00616	Transcription factor	−2.53
	FOSL2	NM_00525	Dimerizes with JUN forming the transcription of factor complex AP-1	−1.65
	Notch3	NM_00871	Transcription factor	−3.64
	RABA1	NM_00416	Member RAS oncogene family	−1.78
Growth factors	INSL4	NM_00219	Member of the insulin superfamily	
	VEGF	NM_00337	Vascular endothelial growth factor	
Kinases	MKNK2	NM_01757	MAP kinase interacting serine/threonine kinase 2	−2.32
	SCAP1	NM_00372	Belongs to the src family kinases	−4.69
Adaptor molecules	NCK1	NM_00615	Adaptor protein involved in transducing signals from receptor tyrosine kinases to downstream recipients such as RAS	−1.67
	SP110	NM_08042	May have a role in the regulation of gene transcription	−2.28
	ABR	NM_02196	These proteins might interact with members of the Rho family	−2.68
	RNF4	NM_00293	RING finger protein, enhances AR-dependent transcription	−3.49
Apoptosis	BCL2A1	NM_00404	BCL2-related protein A1	−1.56
	PDCD4	NM_14534	Thought to play a role in apoptosis	−1.53
	CASP3	NM_00434	Caspase 3 apoptosis-related protease	
Extracellular matrix	ADAM15	NM_00381	A disintegrin and metalloproteinase domain 15 (metargidin)	−1.34
	TIMP1	NM_00325	Tissue inhibitor of MMP1	2.26
	MMP14	NM_00499	Matrix metalloproteinase activates MMP2 protein may be involved in tumor invasion	−1.71
	TIMP2		Tissue inhibitor of MMP 2	−2.71
Cell cycle	S100A10	NM_00296	Calcium-binding, involved in the regulation of cell cycle progression and differentiation	−2.23
	CDK4	NM_00007	Cyclin-dependent kinase 4	−1.75
Proliferation/ Differentiation	DUSP6	NM_00194	Gene product inactivates ERK2,	−1.95
	MAPRE2	NM_01426	Homology with APC suggests involvement in tumorigenesis and proliferative control of normal cells	−1.39
	SHC1	NM_18300	Couples activated growth factor receptors to a signaling pathway	−1.5
Proteases	Kallikrein 10	NM_00277	Serine proteases having diverse physiological functions	−1.72
	KLK5	NM_01242	Kallikrein 5	−1.59
	SPUVE	NM_00717	Protease, serine, 23	
	SLP1	NM_00306	Secreted serine protease inhibitor protects epithelial tissues	−5.04
	CSTA	NM_00521	Encodes a stefin that functions as a cysteine protease inhibotor	−4.65
Cytoskeleton/Motility	DSTN	NM_00687	Actin depolymerizing factor	−1.7
	CLDN1	NM_12110	Integral membrane protein component of tight junctions	−2.45
	CLDN4	NM_00130	Integral membrane protein, which belongs to the Claudine family	−1.33

(*Continued*)

TABLE I. (*Continued*)

Category	Gene	GenBank ID	Description	ACF
	THYB4	NM_02110	Actin sequestering protein, regulates actin polymerization, proliferation migration, and differentiation	−5.1
	LAMC2	NM_00556	Extracellular marrix glycrprotein, implicated in adhesion megration, differentiation, and metastasis	−1.5
	WIRE	NM_13326	Has a role in the WASP-mediated organization of the actin	−4.15
	KRT13	NM_15349	Keratin 13	−4.51
Neuronal	NDN	NM_00248	Mouse studies suggest a role in growth suppression in postmitotic neurons	−1.84
	SST Somatostatin	NM_00104	A regulator of endocrine and nervous system function	−1.97
Metastasis	p8	NM_01238	p8 protein (candidate of metastasis)	−1.94

of the EGFP expressing retrovirus induces the cancer phenotype. The tumors formed in this model retain some key characteristics of human prostate cancer, notably the expression of AR and PSA, and the ability to metastasize.

The c-MYC overexpression combined with a large percentage of infected cells produces a cancer phenotype within our xenograft model that is aggressive and progressive. The cells were obtained from tissue derived from aging males and may already have accumulated genetic hits that, of themselves, are insufficient to alter histology. The levels of c-MYC expressed by a CMV-driven retroviral delivery system would certainly constitute a very large additional molecular hit. The resulting tumors resemble a poorly differentiated advanced carcinoma, however, the growth rate is accelerated in comparison with the human disease. Myc expression can be a pivot point in the cellular decision-making process that determines if a cell will undergo proliferation or apoptosis [50–55]. Myc overexpression within the primary huPrE appears to be exhibiting a proliferative effect demonstrated by the extremely large number of Ki67 positive cells present within the renal capsule graft and the metastatic lesions. The lack of expression of the basal cell markers keratin 14 and p63 indicate that the c-Myc tumor, like human prostate adenocarcinoma, loses basal cells during tumor formation.

Similar c-MYC-based retroviral rodent models created by Thompson and co-workers [25–27,56–59] in prostate and by Edwards [60] in the breast lacked the aggressiveness of our model unless activated ras was also present. Less than 0.1% of epithelial cells carried the retrovirally introduced genes in the mouse TR's while our grafts contained 10%–50% infected cells thus altering the environment considerably as normal cells

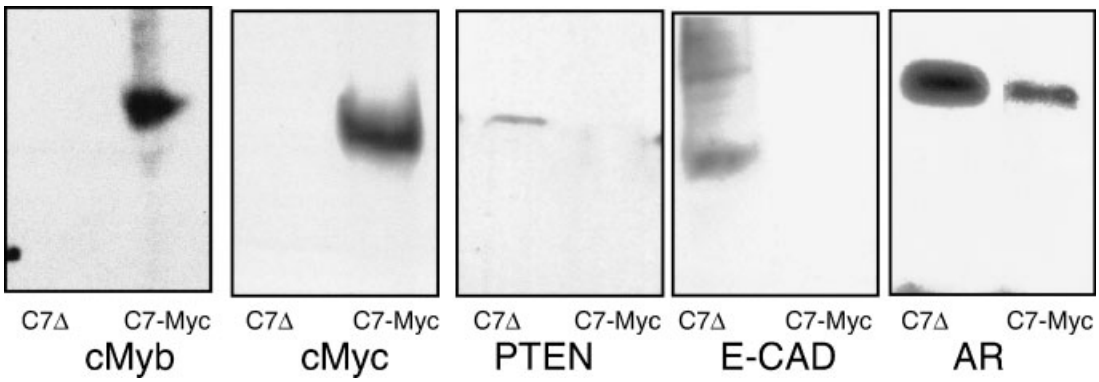

Fig. 5. Western blot analysis of C7-Myc and C7Δ infected cells. Western blot of human prostatic epithelial cells infected with C7-Myc or C7Δ (empty vector). All lanes contain 100 of protein except PTEN (250 μg). EGFP (Top left) is detected only in C7-Myc infected cells, C7Δ infected cells made up approximately 1% of the culture and while fluorescent cells were visible no signal was detected by western analysis. E-cadherin 120 kDa (top middle), Androgen receptor 130 kDa (Top right), cMyb 75 kDa (bottom left) c-MYC 67 kDa (bottom middle) PTEN 60 kDa (bottom right).

have the potential to inhibit the growth of transformed cells [61]. Microarray data do not indicate increased levels of ras mRNA within our C7-Myc tumors. Mouse strain backgrounds modify the effects of the oncogenes myc and ras [27] within the retrovirally infected TR's. By virtue of using human tissue, the present study examines an outbred epithelial cell population.

More recent transgenic mouse studies have demonstrated that the degree of response to c-MYC overexpression in mouse prostatic epithelium is dose related, with increased expression giving rise to more severe phenotypes [20,22]. All three described prostate-specific c-MYC-overexpressing mice develop mPIN. Mice expressing c-MYC using the Probasin promoter progress to invasive cancer in a time frame that is determined by the relative strength of the promoter construct used (ARR_2PB or sPB) [20]. Low levels of c-MYC expression are thought to cause prostatic dysplasia in the prostates of rats treated in the neonatal period with DES [62]. Progression beyond PIN is not observed within the lifetime of the C(3)Myc mouse. As serial recombination experiments using the C(3)-Myc mouse or the DES treated prostates have not been performed it is not known if these PIN-like lesions would progress over time as is thought to happen in humans. Our model possibly shows such an accelerated progression because the epithelium is already primed by both age related genetic changes and environmental/physiological events within the diseased prostate combined with expression of c-MYC from an extremely strong constitutive promoter.

Human prostatic epithelium expresses prostate specific antigen (PSA), in vivo this weak protease belongs to the large family of Kallikreins, and is not, despite the name, totally specific for the prostate. However, PSA is the main basis of the blood tests used to detect and monitor prostate cancer. PSA is androgen-regulated in normal and low-grade prostate cancers, however, as cancers progress to an androgen-independent state, PSA levels climb marking disease progression. Primary cultures of benign prostate cells initially express PSA as do the established cell line LNCaP [63]. However, it is commonly observed that cells in culture rapidly lose expression of steroid receptors, including AR, and consequently lose expression of steroid regulated gene products such as PSA. Tissue recombinants composed of normal prostatic epithelium and rUGM express both AR and PSA [47], as would be expected, since these genes are both regulated by the context in which the cell is growing. More remarkably Myc overexpressing cells express PSA and maintain their androgen receptor expression in culture. Tumors derived from these cells continue to express PSA and AR in vivo adding an important element to the relevance of this model.

Sawyers and co-workers [20] have identified a molecular signature that identifies both the c-MYC overexpressing prostate tumors present in their transgenic mouse model and a subset of human prostate tumors that overexpress Myc. Our cDNA analysis identified several targets that were subsequently analyzed by Western blotting, as our cDNA array differed from that used by the Sawyers group we were unable to compare their molecular signature with the one we obtained. We were however able to use the microarray data to identify proteins previously shown to be involved in tumorigenesis which were regulated in this model. Western blot analysis was used to confirm microarray data on the loss of PTEN and E-cadherin in the c-MYC expressing cells. Both of these proteins are implicated as playing a tumor suppressive role in cancer development and show a marked decrease in expression compared to control cells. Decreased or absent E-cadherin expression is a frequent occurrence in human prostate cancer [64] and this is recapitulated within our myc overexpressing tumor. The undetectable levels (by Western blotting) of E-cadherin present in our myc tumor cells in culture may represent a general loss of adhesion, which would in part explain the low adhesion of the cells both to the tissue culture plastic and other cells resulting in loss of the cobblestone morphology and lack of strong junctions between cells. Immunohistochemistry on the tumor tissue failed to pick up E-cadherin staining on the cell membranes within the tumor (data not shown).

PTEN is a tumor suppressor gene situated at 10q23, a deletion hot spot in prostate cancer [65]. PTEN Knockout mice are embryonic lethal [66] but tissue specific homozygotes (0% gene dose in prostate 100% in most other tissues) and the newer hypomorphic mice (25%–35% gene dose) [67–70] display mPIN that progresses to invasive carcinoma over time. Heterozygote PTEN deletion results in mPIN but requires the presence of a second genetic lesion to progress to an invasive cancer phenotype [71,72]. PTEN encodes a lipid phosphatase that inhibits the PI3 kinase/AKT pathway. The AKT pathway is a pivotal point in the control of cellular homeostasis. Many pathways including TGFβ, IGF, EGF and Ras interact with AKT. The activation of AKT appears to indirectly promote androgen-independent survival of prostate cancer cells, but the mechanism is as yet undetermined [73,74]. PTEN and AKT are upstream of c-MYC; PTEN loss causes upregulation of AKT that in turn upregulates c-MYC. The role of AKT on AR and subsequent repercussions on c-MYC are unknown but the importance of AKT and by extension PTEN in androgen independent growth of prostate cancer correlates with the role of c-MYC.

Microarray data verified by Western blot analysis demonstrated upregulation of the cMyb oncogene in

the C7-Myc model. Myb is usually regarded as a proto-oncogene within the hematopoietic line where it functions as a cell cycle promoter. c-Myb inhibits expression of $p15^{ink4b}$ while transactivating c-MYC, Bcl-2, COX2, IGF-I, and IGF-IR [75–77]. While c-Myb has primarily been studied in the hematopoietic lineage it is also expressed in breast, the gastrointestinal lineage [78], and prostate [79–81], it is upregulated in cancers and some premalignant lesions of these tissues [79,80,82–85]. The upregulation of cMyb in the C7-Myc model may further enhance the transforming ability of c-MYC by stimulating pathways that are not stimulated by Myc.

This work demonstrates the application of retroviral infection strategy followed by tissue recombination to examine the contribution of individual gene products to carcinogenesis. The approach can be used on any of the regulated products within the 8q24 amplicon, or those identified in the cDNA microarray analysis, both alone and in combination to determine their contribution to the final graft phenotype. This in vivo model of prostate cancer based upon human prostatic epithelium has many interesting and potentially useful features. However, the rapid progression and extremely high proliferative rate limits the ability to examine early tumorigenic events. The model retains many characteristics of human prostate cancer including continued expression of AR and PSA. The model also demonstrates downregulation of two tumor suppressors (PTEN and E-cadherin), which are suppressed in human prostate cancer and shows upregulation of Myb, which is known to be upregulated in the human disease. Metastasis is also an important component of the in vivo model. Cell cultures derived from both the initial viral infections and from the tumors retain many of these important characteristics and represent a potentially useful resource to examine prostate cancer biology. We are working to modify the model using a series of weaker constitutive, conditional, and regulatible promoters that will better allow us to follow stages of progression.

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ARTICLE

A Cre Gene Directed by a Human TSPY Promoter Is Specific for Germ Cells and Neurons

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Summary: The testis-specific protein Y-encoded (TSPY) gene is a candidate for the gonadoblastoma locus on the Y chromosome and is expressed in normal testicular germ cells and gonadoblastoma cells of XY sex-reversed females. Although TSPY expression has been demonstrated in gonadoblastoma tissues, it is uncertain if such expression is involved in a causative or consequential event of the oncogenic process. We postulate that if TSPY is involved in gonadoblastoma development, its promoter should be functional in the female gonad before and/or at early stages of tumorigenesis. To test this hypothesis, we generated several lines of transgenic mice harboring a Cre-recombinase transgene directed by a 2.4-kb hTSPY promoter. These mice were crossed with the Z/EG reporter line that expresses EGFP only after a Cre-mediated recombination. Our results showed that hTSPY-Cre;Z/EG double transgenic mice expressed EGFP specifically in the germ cells of both male and female gonads. Further, neurons of the central and peripheral nervous systems also expressed EGFP as early as E12.5 embryonic stage. EGFP was particularly observed in the trigeminal nerve, trigeminal ganglion, dorsal root of the ganglia, and in postnatal and adult brains. These observations support the hypothesis that TSPY plays an active role in gonadoblastoma. The tissue-specific expression of the hTSPY-Cre transgene should also be useful in studies utilizing Cre-mediated gene activation/inactivation strategies in gamatogenesis and/or neurogenesis. genesis 42:263–275, 2005.

Key words: TSPY promoter; gonadoblastoma; germ cell; neurons; Cre recombinase; transgenic mice

INTRODUCTION

The human testis-specific protein Y-encoded (hTSPY) gene was initially identified as a testis-specific gene repeated tandemly >35 times on the short arm of the human Y chromosome (Arnemann *et al.*, 1987; Zhang *et al.*, 1992; Skaletsky *et al.*, 2003). hTSPY is expressed normally in the germ cells of both adult and fetal testes (Zhang *et al.*, 1992; Schnieders *et al.*, 1996; Honecker *et al.*, 2004). It has been postulated to serve a vital function(s) in male germ cell formation and early spermatogenesis (Schnieders *et al.*, 1996; Lau, 1999). Further, TSPY expression has also been observed in germ cells of gonadoblastoma, various forms of testicular tumors, and epithelial cells of prostate cancer (Schnieders *et al.*, 1996; Lau *et al.*, 2000, 2003; Lau and Zhang, 2000). The human TSPY shares significant homology with the SET oncoprotein and is a founding member of the TSPY/SET/NAP-1 protein family (von Lindern *et al.*, 1992; Vogel *et al.*, 1998; Ozbun *et al.*, 2001). Members of this gene family encode proteins that harbor a conserved NAP/SET domain (Ishimi and Kikuchi, 1991) and have been demonstrated to play various roles in cell cycle regulation or cell differentiation (Chai *et al.*, 2001; Seo *et al.*, 2001; Canela *et al.*, 2003). Hence, hTSPY has been hypothesized to regulate the spermatogenic cells in entering male meiosis or in mediating the meiotic divisions (Schnieders *et al.*, 1996; Lau, 1999).

The human TSPY gene cluster has been mapped to the critical region on the Y chromosome harboring the gonadoblastoma locus (GBY), the only oncogenic or tumor-promoting locus currently identified on this male-specific chromosome (Page, 1987; Salo *et al.*, 1995; Tsuchiya *et al.*, 1995). Gonadoblastoma develops most frequently in the dysgenic gonads of XY sex-reversed females and those of girls harboring a mixture of XX/XO and XY gonadal cells. Gonadoblastoma shares significant similarities with the testicular carcinoma-in-situ (CIS) and both have been considered as precursors for the more aggressive germ cell tumors (Jorgensen *et al.*, 1997; Rajpert-De-Meyts *et al.*, 2003). Although the disease mechanism(s) is still uncertain, the aberrant expression of a Y

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chromosome gene(s) in a female gonadal environment has been hypothesized to play an important role in predisposing the dysgenic gonads to tumorigenesis (Page, 1987). TSPY is a strong candidate for GBY locus based on several lines of indirect evidence, including its mapping to the GBY critical region, abundant expression in gonadoblastoma tissues, and significant homology to a family of cell cycle regulatory proteins. However, it is uncertain whether the TSPY expression in gonadoblastoma is a causative event or a consequence of the oncogenic process. Hence, in order to further substantiate its role in gonadoblastoma, we need to demonstrate that its promoter is active in normal or pretumorigenic female gonads. To achieve this goal, we developed a transgenic strategy to investigate the hTSPY promoter activity. We generated several lines of transgenic mice harboring a Cre recombinase gene directed by the promoter of hTSPY gene and evaluated the functionality of the human TSPY promoter by either reverse transcription–polymerase chain reaction (RT-PCR) analysis of the Cre transgene or crossing them with the Z/EG reporter line that expresses EGFP only after a Cre-mediated recombination (Novak *et al.*, 2000). Since EGFP expression can be directly observed as green fluorescence, it provides a convenient means in indirectly detecting the human TSPY promoter activity in tissues and cells of transgenic embryos and adult mice. Our study demonstrated that the hTSPY-Cre transgene was expressed in germ cell lineage in embryos and adult mice of both sexes. In particular, EGFP transgene was activated in adult hTSPY-Cre;Z/EG mice mediated by the hTSPY-Cre gene in the germ cells of both male and female animals, confirming that human TSPY promoter is active in testicular and ovarian germ cells. Significantly, we also detected high levels of EGFP expression in neurons of the central nervous system (CNS) and peripheral nervous system of adult mice. This hTSPY promoter-directed transgene activation could also be observed in selected neurons of fetal brains of mouse embryos as early as E12.5 stage. These latter observations raise the possibility that if similar embryonic expression can be demonstrated in humans, hTSPY might play additional roles in neuronal development in embryos and nervous functions in adults.

RESULTS

Previous studies have demonstrated that a 1.35-kb promoter of the TSPY gene is capable and sufficient in directing a transgene expression in the spermatogonia-derived cell line, GC-1spg (Tascou *et al.*, 2000) and that transgenic mice harboring a human TSPY gene integrated in their Y chromosome show a transgene expression pattern similar to that of humans (Schubert *et al.*, 2003). In the latter study, the transgene harbors a 2.9-kb promoter sequence of the human TSPY gene that is integrated into the Y chromosome of the host. While this transgenic line closely resembles the human TSPY gene in terms of its chromosomal location and expression pattern, it cannot be used in the evaluation of the transgene

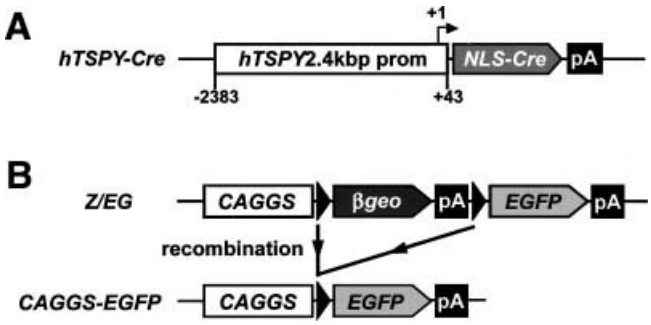

FIG. 1. The Cre-LoxP transgene activation system. **A:** The hTSPY-Cre gene contains a 2.4-kb promoter of the human TSPY gene (white box), Cre recombinase cDNA with a nuclear localization signal (gray arrow), and an SV40 polyadenylation signal (black box). +1 indicates the transcription start site. **B:** The Z/EG responder construct (Novak *et al.*, 2000) consists of two expression cassettes, βgeo and EGFP, directed by a strong CAGGS promoter. Two LoxP sites flank the βgeo gene that is normally expressed in transgenic mice harboring only Z/EG. In hTSPY-Cre;Z/EG double transgenic mice the hTSPY promoter directs the expression of Cre recombinase which subsequently cleaves the sequence flanked by the LoxP sites, thereby repositioning the EGFP gene under the direct regulation of the ubiquitous CAGGS promoter. Hence, EGFP expression is indirectly linked to the hTSPY promoter activity.

expression in the female environment. Based on these results, we selected a 2.4-kb promoter in the present study to identify cells capable of expressing the hTSPY gene in the mouse using the Cre-LoxP gene activation scheme (Novak *et al.*, 2000). Preliminary characterization of this 2.4-kb promoter suggested that it was capable of directing the expression of a reporter gene in the spermatogonia or spermatocytes-derived cell lines, GC-1spg and GC-2spd, respectively, as previously described (Tascou *et al.*, 2000). An expression cassette in which this 2.4 kb promoter of the human TSPY gene was constructed to direct the expression of a Cre recombinase gene (Fig. 1A). Two transgenic founder animals designated #25 (male) and #33 (female) were obtained with this construct using a standard microinjection procedure. The #33 founder when bred with nontransgenic mouse produced offspring with two different copy numbers of the transgene, suggesting the possibility of two integration sites for this founder. Indeed, two sublines from founder #33 were derived, resulting in line 33A (42 copies) and line 33B (6 copies), respectively (Fig. 2A). All transgenes seemed to have integrated into the autosomes of the founding animals, since we did not observe any sex-linked inheritance of the respective transgenes. To determine the expression of the Cre transgene, the animals from lines 25, 33A, and 33B were analyzed by the RT-PCR technique. Line 25 did not show any detectable signal of Cre transgene expression in any tissue (data not shown). However, lines 33A and 33B showed expression of the hTSPY-Cre transgene primarily in the cerebral cortex, cerebellum, and gonads of male and female animals (Fig. 2B,C). These expression patterns are similar to that of a transgenic line harboring a human TSPY transgene on the Y chromosome of the host (Schubert *et al.*, 2003).

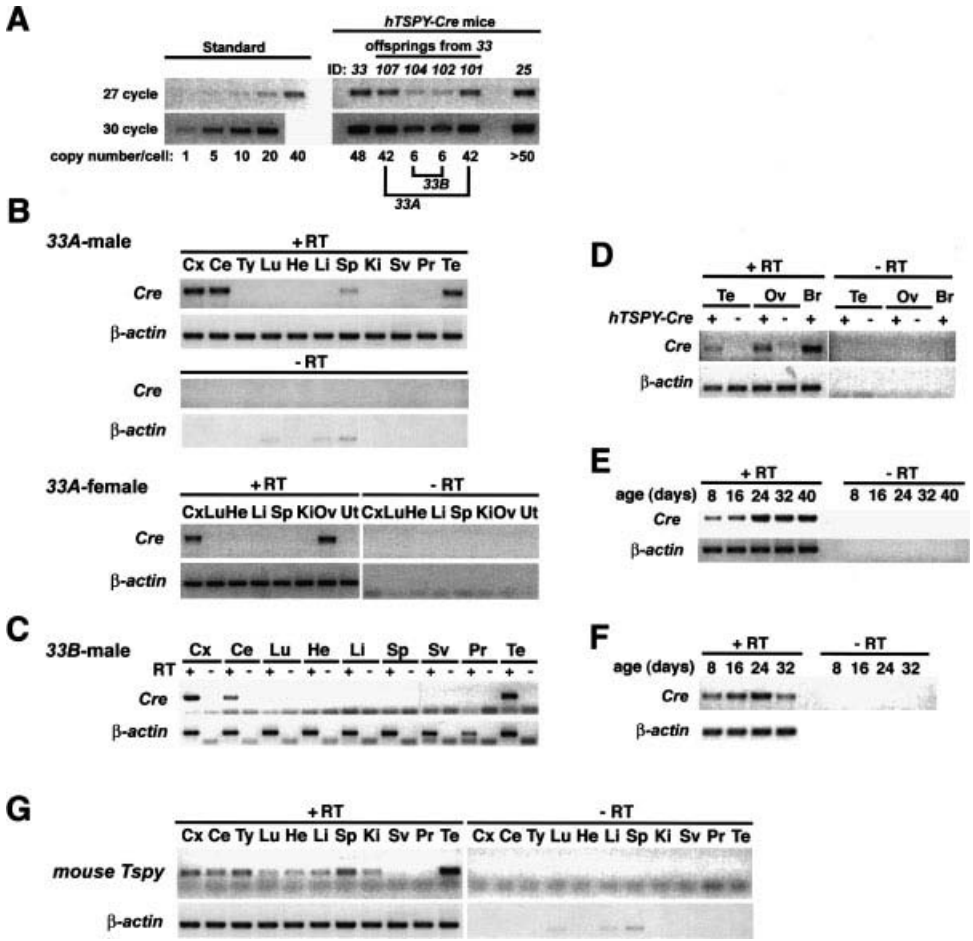

FIG. 2. Estimation of transgene copy numbers and expression analysis by RT-PCR analyses. **A:** Estimation of copy number of hTSPY-Cre transgene in transgenic mice by semiquantitative PCR of tail DNAs. Founder animal 33 had two integration sites that were segregated into two sublines: line 33A (42 copies) and line 33B (6 copies). ID: identity number for individual mouse. **B:** Cre expression patterns in adult hTSPY-Cre transgenic mice from lines 33A (**B**) and 33B (**C**). In both lines 33A and 33B, Cre mRNA was detected only in cerebral cortex, cerebellum, and gonads. Cx, cerebral cortex; Ce, cerebellum; Ty, thymus; Lu, lung; He, heart; Li, liver; Sp, spleen; Ki, kidney; Sv, seminal vesicle; Pr, prostate; Te, testis; Ov, ovary; Ut, uterus. **D:** hTSPY promoter-directed Cre transcripts in the gonads and brains of newborn transgenic mice. Cre transcript was detected in gonads and brains of newborn pups of both sexes. Te, testis; Ov, ovary ; Br, brain. **E:** Cre transcripts in testes of mice at various postnatal ages (days 8–40). **F:** Cre expression in ovary at various postnatal ages (days 8–32). **G:** Expression pattern of the mouse endogenous Tspy. The mouse Tspy transcript was preferentially expressed in testis and at low levels in a wide range of tissues, including the brain. β-Actin was used as a control. +RT indicates the results with reverse transcription reaction; −RT, without reverse transcription reaction. Signals are represented by negative printing of ethidium bromide staining of the RT-PCR products fractionated by agarose gel electrophoresis.

The expression of hTSPY-Cre transgene in the mouse brain was somewhat unexpected. To determine if the expression pattern of the transgene resembled the endogenous mouse Tspy gene, we extended our investigation to the Tspy genes in mouse. The mouse harbors an apparently noncoding Tspy gene on its Y chromosome, due to the presence of several in-frame stop codons (Mazeyrat and Mitchell, 1998). The mouse Tspy transcripts were detected most prominently in the testis and at low levels among other tissues, including the brain (Fig. 2G, mouse Tspy). Hence, the mouse Tspy gene behaves similarly to the hTSPY-Cre transgene in male mice (Fig. 2B,C).

We adopted the Cre-LoxP gene activation system to visualize the hTSPY promoter activity (Fig. 1A,B). In this scheme, animals from the hTSPY-Cre lines were crossed with those harboring the reporter gene, Z/EG, to generate double transgenic mice harboring both transgenes. The hTSPY promoter directed the expression of the Cre recombinase that cleaved the sequence, βgeo gene (i.e. a fusion gene coding for both the β-galactosidase and neo resistant marker), between the loxP sites in the Z/EG transgene, thereby repositioning the EGFP gene directly under the strong CAGGS promoter (Novak *et al.*, 2000). The CAGGS promoter was initially derived from the CAGG (or CAG) promoter (Niwa *et al.*, 1991) consisting of the CMV enhancer and the chicken β-actin promoter, being capable of mediating a high level of expression for the recombined EGFP gene. Hence, EGFP expression indirectly represents the hTSPY promoter activities in these double-transgenic mice. Using this strategy, we

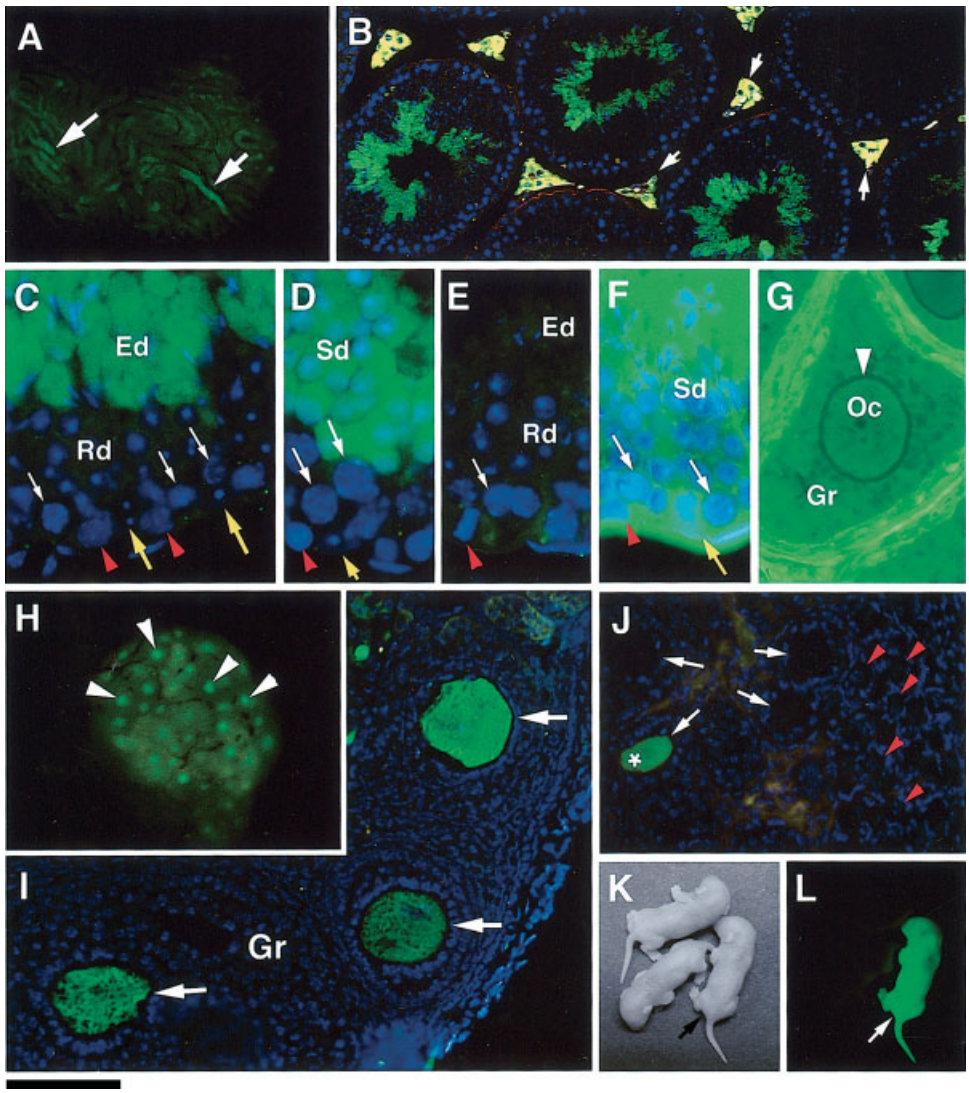

FIG. 3. Expression of EGFP in gonads of hTSPY-Cre;Z/EG double-transgenic mice. **A:** EGFP expression could be observed directly in dissected hTSPY-Cre;Z/EG adult testis (arrows). **B–D:** EGFP expression in male germ cells of testis in hTSPY-Cre;Z/EG mice generated by crossing 33A (**C**) and 33B (**D**) with the Z/EG line. DNA was counterstained by DAPI (blue). Interstitial tissues including the Leydig cells showed yellow fluorescence that was not part of the EGFP signal (arrows in **B**). **E:** No EGFP expression was observed in the testis of a single transgenic Z/EG mouse. Orange arrowheads indicate spermatogonia; yellow arrows, Sertoli cells; white arrows, spermatocytes; Rd, round spermatids; Ed, elongated spermatids. **F,G:** Testis (**F**) and ovary (**G**) sections of F2 animals showing ubiquitous expression of the fully recombined CAGGS-EGFP reporter, respectively. **H:** Direct observation of EGFP fluorescence from a dissected ovary from an 8-day-old hTSPY-Cre; Z/EG mouse. EGFP positive oocytes were identifiable (arrowheads). **I,J:** EGFP expression in maturing oocytes (arrows) of adult (**I**) and 8-day old (**J**) ovaries of hTSPY-Cre;Z/EG mice. DNA was counterstained by DAPI (blue). Granulosa cells (Gr) were EGFP negative. Arrowheads (in **J**) indicate oocytes in primary follicles; white arrows indicate oocytes in late primary and early secondary follicles; asterisk, an EGFP-positive oocyte. **K,L:** Pups from a hTSPY-Cre;Z/EG female mouse mated with a nontransgenic male mouse. Pups harboring the recombined CAGGS-EGFP transgene could be identified by their EGFP fluorescence (**K**, room light, and **L**, blue excitation light). Scale bar = 115 μm in **B**; 34 μm in **C–E**; 100 μm in **I**; 83 μm in **G**.

tested both lines 33A and 33B and showed that both were capable of inducing similar Cre-mediated recombination and activation of the EGFP transgene in the hTSPY-Cre;Z/EG double transgenic mice. Since line 33A harbored a higher number of transgene and the intensity of its expression was also higher than that of line 33B, line 33A was primarily used in the analyses described below.

The expression of the EGFP reporter was readily observed directly in dissected adult testis of hTSPY-Cre;Z/EG double transgenic mice (Fig. 3A). Immunofluorescence analysis showed that EGFP was primarily detected in round and elongating spermatids (Fig. 3B–D) in double-transgenic mice derived from crossing of the Z/EG animals with those of Line 33A (Fig. 3C) and Line 33B (Fig. 3D). However, somatic cells, spermatogonia, and spermatocytes did not show EGFP expression (arrows in Fig. 3C,D), suggesting that the Cre-mediated recombination might have occurred around the second

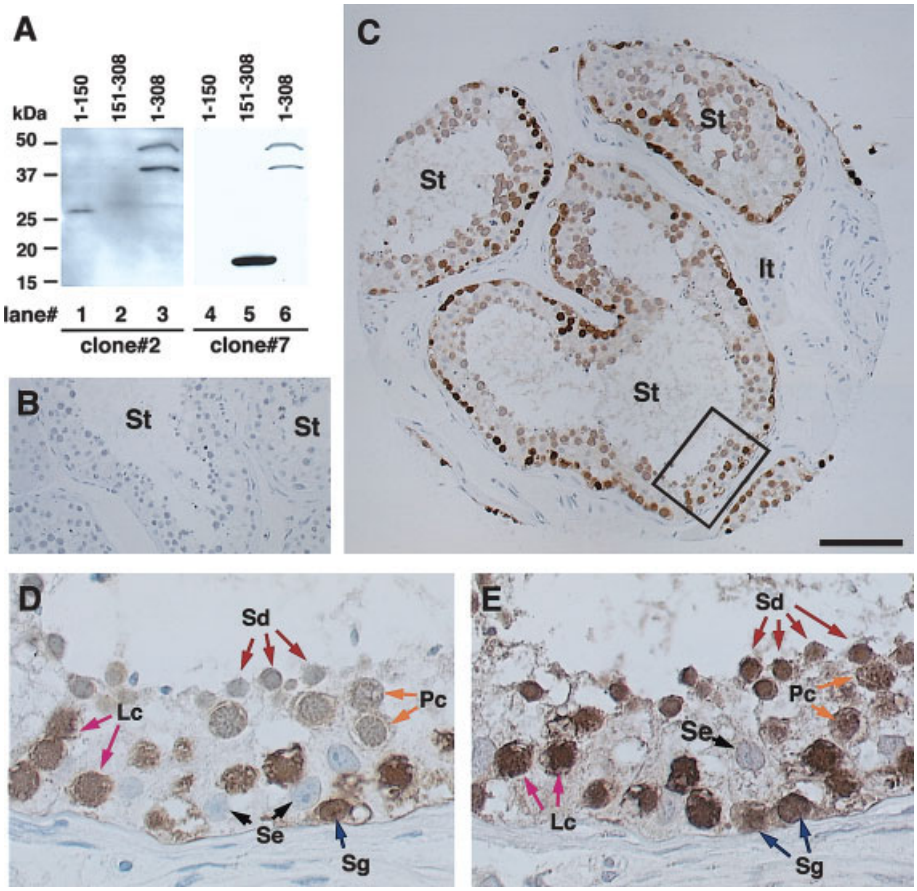

FIG. 4. TSPY expression in normal human testis. **A:** Western-blotting of protein extracts of COS7 cells transfected modular genes coding for different domains of TSPY. Antibodies from hybridoma clone #2 and clone #7 showed specificities for N-terminal (residue 1–150, lanes 1, 4) and C-terminal (residue 151–308, lanes 2, 5) halves of the human TSPY, respectively. The phosphorylated full-length (residues 1–308, lanes 3, 6) TSPY is ~38-kDa in the testis (Schnieders *et al.*, 1996) was detected by both monoclonal antibodies. The larger 48-kDa band in lanes 3 and 6 could be result(s) of additional posttranslational modification of the protein by the COS7 cells. **B–E:** Images of normal human testis immunostained by mouse pre-immune serum (**B**), and clone #2 (**C,D**), and clone #7 (**E**) antibodies, respectively. **D,E:** Magnified images of the boxed area in **C**, stained by clone #2 (**D**) and clone #7 (**E**), respectively. Most germ cells from spermatogonia to spermatids were immunoreactive to both clone #2 and clone #7 (Sg, Lc, Pc and Sd in **D,E**), while the somatic cells including Sertoli cells and Leydig cells were negative. However, clone #7 stained both the spermatogonial cells and the spermatids with equal intensity, while clone #2 showed a preferential staining of the spermatogonial cells. St, seminal tubules; It, interstitial cells; Se, Sertoli cells; Sg, spermatogonia; Lc, leptotene spermatocytes; Pc, pachytene spermatocytes; Sd, spermatids. Scale bar = 100 μm in **B,C**.

meiotic division. This observation is somewhat different from the previously reported human situation in which TSPY is specifically expressed in spermatogonia (Schnieders *et al.*, 1996). It is conceivable that the recombination in these hTSPY-Cre;Z/EG mice might have occurred at earlier stages, e.g., spermatogonia and/or spermatocytes, but the levels of recombination were insufficient to activate the EGFP gene and/or CAGGS promoter could become most active in later stages, thereby delaying the expression of the EGFP reporter at the round and elongated spermatids. Alternatively, TSPY could be expressed in other germ cells, such as the spermatids, in addition to the spermatogonia, but the available TSPY antibodies are incapable of detecting its expression in later stages of spermatogenesis.

To resolve these two possibilities, we reexamined the expression of TSPY in normal human testis using immunohistochemistry with two monoclonal antibodies generated against a full-length recombinant human TSPY protein. The antibodies from two hybridoma lines, designated as clone #2 and clone #7, recognized the N-terminal (residue #1–150) and C-terminal (residue #151–308) halves of the human TSPY protein respectively (Fig. 4A). Clone #7 stained positively for most germ cells, but preferentially for the spermatogonia and round spermatids (Fig. 4E). However, clone #2 stained preferentially the spermatogonial cells (Fig. 4C,D). The somatic cells, including Sertoli and Leydig cells, were negative for both antibodies. These findings, specifically the expression of TSPY protein in the germ cells at late stages of spermatogenesis, differ from previous reports of TSPY expression in the spermatogonial cells of the human testis (Schnieders *et al.*, 1996). The differential detection of TSPY proteins by these two N- and C-terminus-specific

monoclonal antibodies is an interesting observation. Recent studies demonstrated that several minor TSPY transcripts (designated Exon1A, 1B, and 1C) could be derived from alternative splicing of cryptic splice sites located at the first exon, resulting in in-frame deletions of 87, 104, and 139 amino acids from the N-terminal half of the TSPY protein (Lau *et al.*, 2003). Since clone #2 is specific for the N-terminus, variant proteins lacking this domain, as encoded by Exon1A–1C transcripts, will not be recognized by this monoclonal antibody. On the other hand, clone #7 is specific for the C-terminal half of the protein, present in all TSPY isoforms, and is capable of detecting most TSPY proteins. The present results suggest that the abbreviated forms of the TSPY protein could be preferentially expressed in round spermatids, while both the full-length and the abbreviated forms could be expressed in the spermatogenic cells. These findings suggest that TSPY expression in different stages of the spermatogenic cells in the human testis may be more complex than what was originally observed.

The Cre transcript levels were also evaluated in the testes of single hTSPY-Cre transgenic mice from newborn (day 1) to 40 days after birth by semiquantitative RT-PCR. Our results showed that the Cre recombinase transcripts were present in newborn male testis, ovary, and brain (Fig. 2D), but not in nontransgenic littermates. The level of Cre transcripts increased in the testis and became most abundant at day 24, when meiotic II division was initially observed and round spermatids appeared (Fig. 2E, Cre). Our results, together with recent observations of TSPY expression in prespermatogonial cells in both fetal and postnatal human testes (Honecker *et al.*, 2004), suggest that the human TSPY promoter is active in most gonocytes and prespermatogonia at fetal and postnatal testes and early and late stages of spermatogenesis in adult testis.

The ovarian expression of the Cre transgene could be detected with RT-PCR as early as newborn (Fig. 2D) and throughout the postnatal stages (Fig. 2F). When these hTSPY-Cre transgenic mice were crossed with the Z/EG reporter line, EGFP could be observed directly at the follicles in ovaries of 8-day-old double-transgenic mice (Fig. 3H, white arrowheads). EGFP was primarily located in the oocytes in the late primary follicles (Fig. 3J, white arrows) and those of maturing secondary follicles of adult females (Fig. 3I, white arrows). However, EGFP was not observed in the granulosa cells (Fig. 3I, Gr) nor the early primary follicles (Fig. 3J, orange arrowheads) in these hTSPY-Cre;Z/EG mice. The expression of EGFP in either the oocytes or sperm of these hTSPY-Cre;Z/EG mice did not seem to affect their fertility nor reproductive functions. The recombined CAGGS-EGFP gene could be transmitted to the offspring when these double-transgenic mice were mated with nontransgenic partners. Since recombined EGFP transgene was under the direction of the CAGGS promoter, it is expressed ubiquitously among many tissues of positive pups of the F2 generations (Fig. 3K,L). Although the Cre transgene could also be expressed in early primary oocytes and spermatogonia and spermatocytes, since its transcript could be detected in the ovaries and testes of newborn and prepuberty animals, respectively, by RT-PCR (Fig. 2D,E), the expression of EGFP reporter in the double transgenic mice is probably a true indication of the actual activities of the Cre recombinase directed by the human TSPY promoter. Analysis the F2 animals harboring the recombined CAGGS-EGFP transgene showed that EGFP was expressed almost ubiquitously in most cells in both the testis and the ovary (Fig. 3F,G, respectively), hence the differential expression of EGFP cannot be attributed to any differences in CAGGS promoter activities in the respective cell types in the hTSPY-Cre;Z/EG double transgenic mice. These results support the postulation that the 2.4-kb human TSPY promoter is functional in germ cells of postnatal testis and ovary, and is most active in germ cells at late meiotic stages.

Detection of Cre transcripts in brains of hTSPY-Cre mice was an interesting observation (Fig. 2B,C). Although TSPY transcripts have been reported in human brain EST databases (GenBank access. nos. BI828033, BX281192) and in transgenic mice harboring a human TSPY transgene on their Y chromosome (Schubert *et al.*, 2003), the expression of TSPY in the brain has not been characterized in detail. The availability of the hTSPY-Cre transgenic mouse offers an opportunity to evaluate the behavior of the human TSPY promoter in directing expression of the EGFP reporter gene in the brain. The frontal view of the whole brain from hTSPY-Cre;Z/EG transgenic mice showed a general green fluorescence

FIGURE 5. EGFP expression mediated by hTSPY-Cre recombination in adult brain. **A–D:** Frontal (**A,C**) brain and brain stem views (**B,D**) of double-transgenic and single transgenic mice respectively were observed under the regular room light (left panels) and EGFP excitation light (right panels). Only brains of double-transgenic mice showed EGFP expression. Yellow arrowhead, the trigeminal nerve; orange arrows, anterior lobes of pituitary gland in **B,D**. **E:** A section of the cerebral cortex; no cell was double-stained by anti-GFP (green) and anti-GFAP (red), indicating that these two proteins did not express in the same cells. **F–H:** Sections of the dorsal cortex of the inferior colliculus (DCIC), the molecular layer (MOL), and the granule layers (GRN) of the cerebellum, showing EGFP (**F**) and NeuN (**G**) expression. **H:** Merged image of **F** and **G**, showing a colocalization (orange color) of both EGFP and NeuN in the same cells. White arrows point to Purkinje cells that are negative for NeuN antibody. **I–N:** Sections of the hippocampus showing EGFP (**I**) and NeuN (**J**) expression, respectively. The boxed areas in **I** and **J** are magnified in **K** and **L**, respectively. **M,N:** DNA (blue) staining and merged images of the boxed areas, respectively. Samples presented in **F–N** were derived from a female hTSPY-Cre;Z/EG double transgenic mouse. **O–R:** hTSPY-Cre33B;Z/EG also showed EGFP expression in neuron-specific manner. EGFP expression (**O,Q**) and merged image with NeuN expression (**P,R**) in cerebellum (**O,P**) and pyramid cell layer of hippocampus (**Q,R**), respectively. Arrows indicate Purkinje cell (**F,H,O,P**). Only NeuN-positive neurons, except Purkinje's cells (arrows in **G,P**), expressed the EGFP reporter and were observed as yellow/orange in the merged images (**H,N,P,R**). All figures, except **O–R**, were obtained from hTSPY-Cre33A;Z/EG double transgenic mice, while **O–R** were derived from hTSPY-Cre33B;Z/EG mice. CA1-CA3, fields of the hippocampus; DG, dentate gyrus; cortex, cerebral cortex; Or, oriens layer of hippocampus; Py, pyramidal cell layer of hippocampus; Rad, stratum radiatum of hippocampus. Scale bar = 50 μm in **E,Q,R**; 100 μm in **F–H** and **O,P;** 126 μm in **K–N**.

(Fig. 5A). At the brain stem, green fluorescence was observed in the optic cord and trigeminal nerve (Fig. 5B, yellow arrowhead), but not in anterior pituitary gland (Fig. 5B, orange arrowhead). Single transgenic mice harboring only the Z/EG transgene did not show any green fluorescence at the same brain structures (Fig. 5C,D).

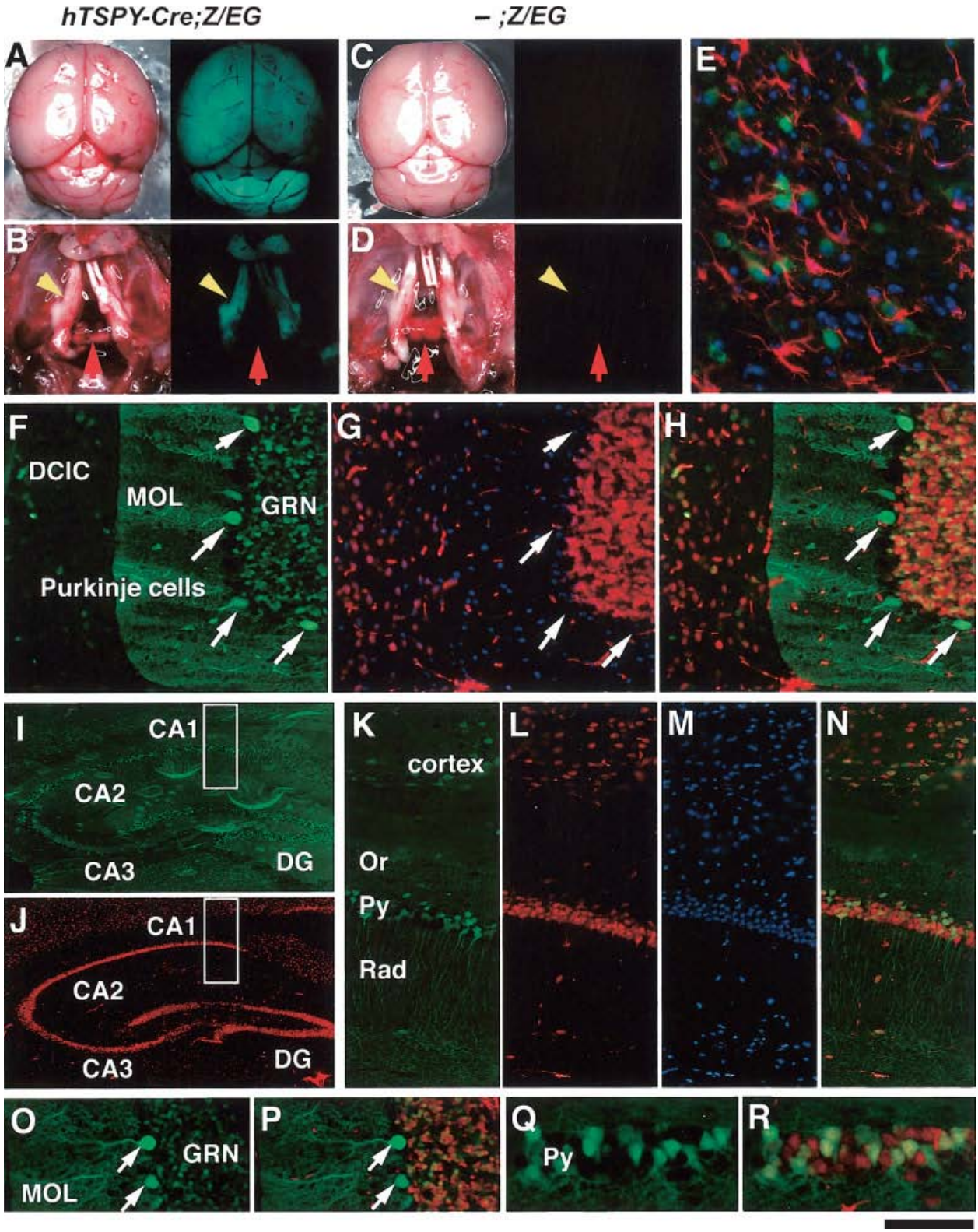

FIG. 5

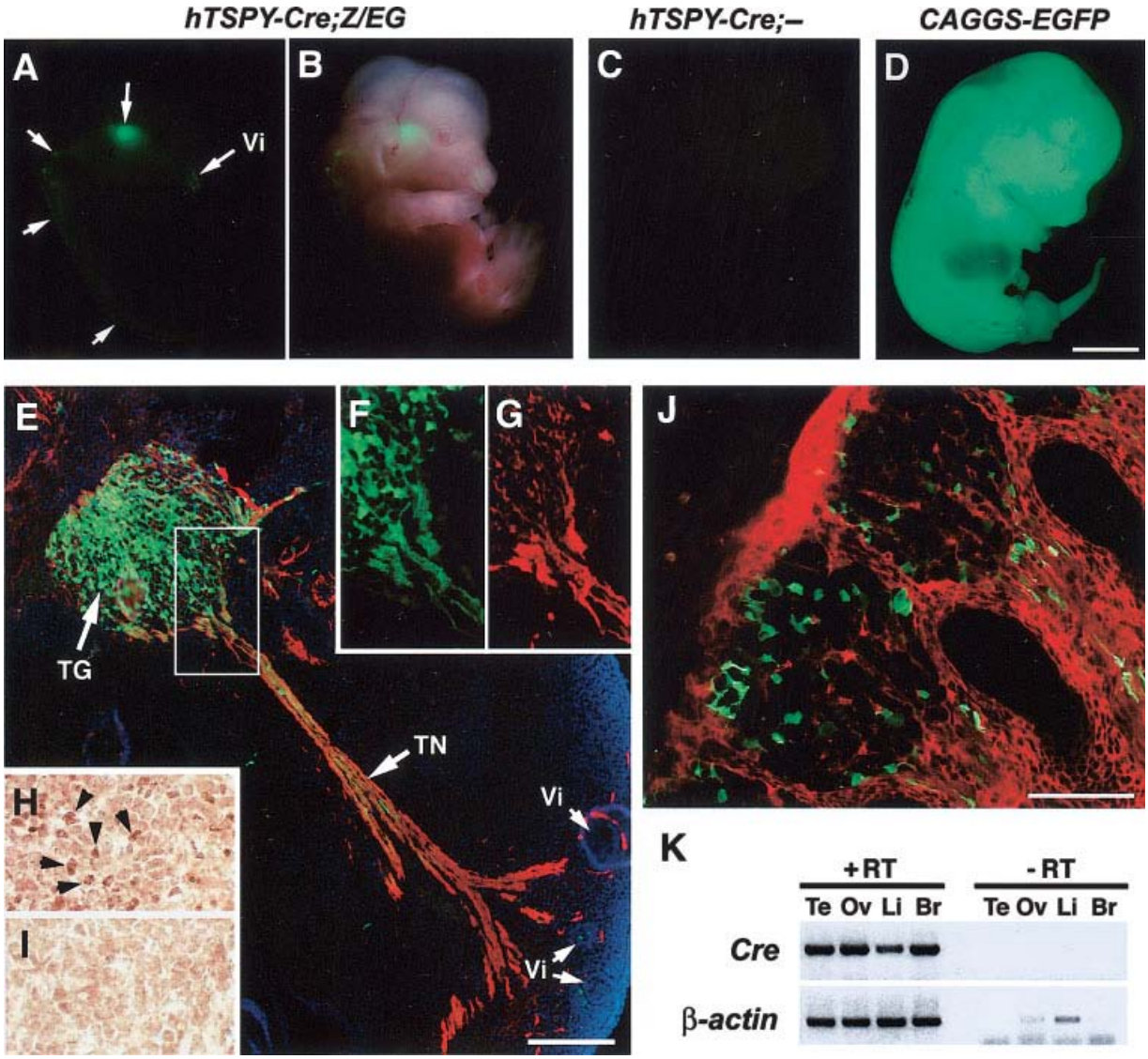

FIG. 6. EGFP and Cre transgene expression in E12.5 hTSPY-Cre;Z/EG embryos. **A–D:** Embryos observed under EGFP excitation light. **A,B:** A hTSPY-Cre;Z/EG embryo. **B:** Merged image of that in **A** and one under room light. Green fluorescence was observed only around the nerve systems (arrows). Vi indicates follicles of vibrissa. **C:** A single hTSPY-Cre transgenic embryo showing no green fluorescence. **D:** An F2 embryo harboring a fully recombined CAGGS-EGFP expressed EGFP ubiquitously. **E–G:** Immunofluorescence of section at the head area (similar to that in **A**) of a female hTSPY-Cre;Z/EG embryo. **E:** Merged image of EGFP (green), neurofilament-M (red), and DNA (blue). **F,G:** Individual images of EGFP (**F**) and neurofilament-M (**G**) of boxed area in **E**. **H,I:** Cre expression in trigeminal ganglion detected by anti-Cre antibody staining. Cre expressing cells were detected only in hTSPY-Cre;Z/EG embryo (**H**, arrowheads), but not in –;Z/EG embryo (**I**). **J:** Section of DRG from a male hTSPY-Cre;Z/EG embryo immunostained by anti-EGFP (green) and anti-β-galactosidase (red). Cells that had undergone a Cre-mediated recombination showed EGFP expression, i.e., in the sensory neurons, and those that did not go through such a recombination showed β-galactosidase expression. **K:** hTSPY promoter activities in hTSPY-Cre tissues of E12.5 embryos detected by RT-PCR method. Cre expression was found in gonads, liver, and brain. Abbreviations are the same as in Figure 2. Scale bars = 2 mm in **A–D;** 200 μm in **E;** 100 μm in **J**.

To characterize the hTSPY promoter activity in the brain, we analyzed the EGFP expression in conjunction with immunofluorescence staining against the neuronal-specific nuclear protein (NeuN) and astrocyte-specific glial fibrillary acidic protein (GFAP) (Debus *et al.*, 1983; Mullen *et al.*, 1992). The cerebellum and the dorsal cortex of the inferior colliculus (DCIC) contained only NeuN-positive cells, while Purkinje cells were positive for only EGFP (Fig. 5F–H). The ratio of EGFP-positive cells to all NeuN-positive cells was slightly different among the hTSPY-Cre;Z/EG mice; however, there was no significant difference between male and female animals (data not shown). Similar results were observed in the cerebral cortex and the hippocampus (Fig. 5I–N) of the double-transgenic mice, while EGFP did not show any colocalization with GFAP-positive cells (Fig. 5E). As shown in hTSPY-

Cre33A;Z/EG mice, the hTSPY-Cre33B;Z/EG mice also showed EGFP expression in a neuron-specific manner (e.g., Fig. 5O–R). These results suggest that the hTSPY promoter is specific for neurons, but not astrocytes, in the brain.

Sexual dimorphism has been postulated to play a role in brain development during embryogenesis (MacLusky and Naftolin, 1981; Arnold and Burgoyne, 2004; De Vries, 2004). The availability of the hTSPY-Cre and Z/EG mice provided an opportunity to determine the promoter activities of a human Y chromosome gene during mouse embryogenesis. Embryos at or immediately after sex determination, i.e., at stages E12.5–13.5, were used in the present studies. At this time gonadal sex has already been decided by the action of the Sry gene while the sexual hormonal environments are still not fully developed (MacLusky and Naftolin, 1981; Arnold and Burgoyne, 2004; De Vries, 2004). RT-PCR analysis of total RNAs derived from testis, male liver and brain, and ovary of E12.5 hTSPY-Cre embryos showed detectable Cre transcripts (Fig. 6K). At E12.5, green fluorescence was visible directly at the head and vertebral column of both male and female double-transgenic hTSPY-Cre;Z/EG embryos (Fig. 6A,B, white arrows) while it was absent in single transgenic hTSPY-Cre embryos (Fig. 6C). Immunofluorescence staining of tissue sections from these embryos showed that EGFP was expressed principally in the trigeminal ganglion (TG) and partially in the trigeminal nerve (TN) of the primitive brain (Fig. 6E), which were also positive for neurofilament M (Fig. 6E–G). Immunostaining of similar sections showed that they were also positive for the Cre protein (Fig. 6H). These results suggest that the human TSPY promoter is most active in embryonic neurons at these developmental stages of embryogenesis.

Although Cre recombinase transcripts were detected in various embryonic tissues at this developmental stage, EGFP, indicative of an efficient recombination, was not easily observed among these tissues, including both the testis and ovary. To evaluate the recombination status of the Z/EG transgene in the double-transgenic embryos, we analyzed the expression of both EGFP and βgeo genes in these tissues, detected by antibodies against EGFP and β-galactosidase, respectively. Our results demonstrate that neurons in the dorsal root ganglion (DRG) underwent successful recombination and showed high EGFP expression (Fig. 6A, white arrows, and 6J), while the EGFP reporter transgene in most surrounding nonneuronal cells did not undergo any recombination and remained β-galactosidase-positive. Morphologically, many of these EGFP-positive cells seemed to be sensory neurons of the peripheral nervous system. The fetal gonads, however, showed little or no EGFP immunofluorescence and most cells were still positive for β-galactosidase (data not shown). These results suggest that insufficient Cre recombinase was available for an efficient recombination in the fetal gonads, and perhaps other tissues (e.g., liver), despite the presence of the Cre transcripts (Fig. 6K).

DISCUSSION

The GBY locus has been hypothesized to harbor a gene(s) that serves a vital function in the testis; however, when present in a female gonad it exerts an oncogenic function leading to gonadoblastoma development (Page, 1987; Lau, 1999; Tsuchiya *et al.*, 1995). For the past several years the human TSPY gene has been considered a strong candidate for the GBY locus, based on its mapping to the GBY critical region on the human Y chromosome and expression in tumor germ cells of gonadoblastoma samples and in male-specific cancers, testicular seminoma, and prostate cancer (Lau, 1999; Lau *et al.*, 2000, 2003). Although TSPY encodes a protein(s) harboring an NAP/SET domain capable of binding to type B cyclins (Kellogg *et al.*, 1995), the exact mechanism of its postulated oncogenic or tumor-promoting properties has not been resolved. Significantly, it is uncertain whether its expression in gonadoblastoma tissue represents a causative or consequential event. If it plays an oncogenic or tumor-promoting role, we surmise that it should be expressed in female germ cells, prior to or at the early stages of gonadoblastoma development. The present study demonstrates that the 2.4-kb human TSPY promoter, when integrated into the autosomes of transgenic mice, is capable of directing the expression of a modular Cre gene in the female germ cell lineage. Its expression increases significantly in the late primary and secondary oocytes of the ovaries, thereby efficiently activating the EGFP reporter in the Cre-LoxP system used in this study. Hence, it is possible that TSPY could be similarly active in the germ cell lineage of XY sex-reversed females before or at the early stages of the oncogenic process, thereby supporting the hypothesis that TSPY plays a causative or predisposing role in gonadoblastoma development in these patients.

TSPY expression has been observed in the gonocytes and prespermatogonia in human fetal and postnatal testes (Honecker *et al.*, 2004) and spermatogonia and spermatocytes in adult testis (Schnieders *et al.*, 1996). Similar expression in adult testis has been reported in transgenic mice harboring an 8.2-kb genomic fragment encompassing the entire human TSPY gene on the mouse Y chromosome (Schubert *et al.*, 2003). However, our results clearly demonstrate that the human TSPY promoter is active in both early and late stages of spermatogenesis in the mouse. Reexamination of TSPY expression pattern in adult human testis using a panel of domain-specific monoclonal antibodies suggests a bimodal expression pattern of TSPY isoforms initially in early spermatogonia and spermatocytes and then in late spermatids (Fig. 4). We surmise that the discrepancy in detecting TSPY protein in various stages of spermatogenesis between the laboratories could be attributed to the differences in the specificities of the antibodies used in the respective studies. Unfortunately, the single-copy Tspy gene on the mouse Y chromosome harbors several in-frame stop codons in its coding sequence and does not encode any functional protein and, therefore, could

not be used in expression studies of the endogenous Tspy protein in the present study (Mazeyrat and Mitchell, 1998). The rat, however, possesses a functional Tspy gene on its Y chromosome. Using a rat Tspy-specific polyclonal antibody, we were able to detect the rat Tspy protein in the spermatids of adult rat testis (Kido and Lau, unpubl. data). This latter observation suggests that the Tspy gene might be active in the spermatids of the rodents. If the regulatory mechanism in the rat is preserved in the mouse, our results suggest that the 2.4-kb human promoter is properly regulated in the spermatids of transgenic mice.

Studies on TSPY expression, initially postulated to be testis-specific, in the brain are somewhat limited, despite the fact that transcripts of the human TSPY transgene could be detected by the Northern hybridization technique (Schubert *et al.*, 2003) and TSPY ESTs have also been reported in databases of brain cDNA libraries (GenBank access. nos. BI828033 and BX281192). The expression of the human TSPY promoter directed Cre recombinase gene, and that of the EGFP reporter gene, in the neurons of both central and peripheral nervous systems of transgenic mice is a significant finding. Using the Cre-LoxP gene activation system, we are able to demonstrate the specific cell types in the central and peripheral nervous systems where the TSPY promoter is active. In particular, the neuron-specific expression of the EGFP reporter in the trigeminal ganglia, trigeminal nerve, and dorsal root ganglia of E12–13 mouse embryos suggests that the human TSPY gene promoter is active at the time or immediately after sex determination in these embryos.

Currently, it is uncertain which cis-elements are involved in germ cell and neuron-specific expression of the human TSPY promoter. The human TSPY gene contains various CpG islands within its promoter and first exon sequences. In fact, these CpG islands were used as functional landmarks in identifying the TSPY functional gene from a human Y chromosome cosmid library (Zhang *et al.*, 1992). Several germ cell-specific genes have been demonstrated to harbor CpG islands that are regulated through various methylation, demethylation process(es), and recruitment of transcription factors (De Smet *et al.*, 1999; Iannello *et al.*, 2000; Hisano *et al.*, 2003). The human TSPY promoter harbors a 5′-GGGTGGG-3′ motif at 108 bp upstream of its transcription start site. This element has also been detected on the promoters of some testis-specific genes, including proacrosin, protamine 1 and 2, and Hsp70-3, and hence could potentially play a key role in determining the germ cell-specific expression of the TSPY gene (Friedrich *et al.*, 1998; Steger, 1999). Currently, we are uncertain if any enhancer element for spermatogonial cells is present in the 2.4-kb TSPY promoter. Since animals from the transgenic line harboring the 8.5-kb (including a 2.9-kb promoter) TSPY transgene (Schubert *et al.*, 2003) express their transgene in spermatogonial cells, as detected by immunostaining with a TSPY antibody generated by these investigators, one might argue that any spermatogonial enhancer elements could likely be present within the 500-nucleotide sequence between −2.9 kb to −2.4 kb of the human TSPY promoter.

Alternatively, the difference in expression between the two studies could reflect the effects of meiotic sex chromosome inactivation (MSCI). During early meiotic prophase, the X and Y chromosomes are subjected to chromosome-wide silencing and for many genes it persists until the end of spermatogenesis. By contrast, the autosomes escape this silencing mechanism (Tuner *et al.*, 2005). Thus, the TSPY transgene that integrated on the Y chromosome of the host (Schubert *et al.*, 2003) will be subjected to MSCI and therefore be silent in late spermatogenesis, while those integrated on the autosomes (such as the hTSPY-Cre transgenes in the present study) will escape MSCI and will therefore be expressed at late spermatogenic stages.

The neuron-expression of the TSPY promoter is somewhat unclear. The Olf-1 transcription factor has been postulated to regulate the olfactory receptor gene expression in adult and during neuronal development, by binding to the Olf-1 binding motif, 5′-TCCYYRRGGAG-3′, present in its target genes (Wang and Reed, 1993). The human TSPY promoter harbors such a motif at 533 bp upstream of its transcription initiation site. This element could be responsible for the hTSPY-Cre activation of the EGFP reporter in the trigeminal ganglia, trigeminal nerve, and sensory neurons of E12.5 embryos and in the brains of adult mice. Although TSPY has been hypothesized to be critical in germ cell development and male meiosis, its roles in neural development and brain function is clearly understood. However, a recent study of the sudden infant death with dysgenesis of the testis syndrome (SIDDT) detected a mutation of the TSPY-like 1 gene (TSPYL1), an autosomal analog of the TSPY gene (Puffenberger *et al.*, 2004). The mutated TSPYL1 gene encodes a protein with a deleted TSPY/NAP/SET domain, conserved among members of this gene family. This finding and those of the present study suggest that TSPYL1 and TSPY may play a role(s) in neuronal development and/or functioning of the nervous system, in addition to its function as a germ cell factor in the testis. The hTSPY-Cre lines should be useful for studies requiring germ cell and neuron-specific gene activation or gene deletion with the Cre-LoxP system in transgenic mice.

MATERIALS AND METHODS

Animals

The Z/EG reporter mouse (Novak *et al.*, 2000) was obtained from the Jackson Laboratory (Bar Harbor, ME; stock number 003920). FVB mice (Charles River Laboratories, Wilmington, MA) were used in maintaining the animals. Mice older than 6 weeks were analyzed as adults. All transgenic mice used in this study were heterozygous for the respective transgene. The Institutional Animal Care and Use Committee of the VA Medical Center approved all experimental procedures in accordance with the NIH *Guide for Care and Use of Laboratory Animals*.

Construction of the phTSPY-Cre Plasmid and Generation of Transgenic Mice

The 2.4k-bp promoter region (−2383 to +43 from the transcription start site) of human TSPY gene was excised by SphI from the plasmid, pTSPY12.5 (Zhang *et al.*, 1992), and subcloned as a blunt-end fragment into the XhoI site of pGL2-basic plasmid (Promega, Madison, WI), resulting in the plasmid pGL2-hTSPY2.4. The CMV promoter in the expression plasmid pCS2plus (Rupp *et al.*, 1994; Turner and Weintraub, 1994) was replaced by the hTSPY2.4 promoter, resulting in an expression vector, phTSPY2.4, consisting of a hTSPY2.4 promoter, multiple cloning site, and polyadenylation site from SV40. The Cre gene was excised from pCAGGS-NLS-Cre-PGKPuro/cg (a gift of C. Lobe, Sunnybrook and Women's College Health Science Center, Toronto) by PstI and BglII, and inserted into the HindIII site of phTSPY2.4. The resultant phTSPY-Cre plasmid was linearized by ScaI and gel-purified by binding to glass beads (BIO 101, La Jolla, CA). The fragment was microinjected into the pronuclei of one-cell embryos of B6CBAF1 and processed for transgenic mouse construction using standard procedures by the Stanford Transgenic Mouse Core Laboratory. All offspring were analyzed by PCR on genomic DNA from tail biopsies with Cre primers (Cre-2F: 5′-TGC ATT ACC GGT CGA TGC AA-3′, Cre-3R: 5′-AGC TAC ACC AGA GAC GGA AA-3′) and EGFP primers (EGFP-F: 5′-GCA AGC TGA CCC TGA AGT TCA TC-3′, EGFP-B: 5′-AAC TCC AGC AGG ACC ATG TGA TCG-3′). A semiquantitative PCR procedure was used to estimate the transgene copy numbers with 0.1 μg of genomic DNA from tail biopsies at 27 and 30 cycles, using phTSPY-Cre plasmid as a reference.

Semiquantitative RT-PCR

Total RNA was isolated from various tissues by the TORIZOL reagents (Invitrogen, Carlsbad, CA) or RNA easy mini kit (Qiagen, Valencia, CA) according to protocols supplied by the respective vendors for adult stages and newborn stages, respectively. After treatment by RQ1-DNase (Promega) to remove any contaminated DNA, 0.36 μg of total RNA (final 21 μl reaction) was reverse-transcribed with the SuperScript reverse transcriptase kit 2 (Invitrogen). One μl of reverse-transcribed product was subjected to PCR using the touchdown procedure: 1 × 95°C, 5 min; 2 × (95°C, 1 min; 68°C, 1 min; 72°C, 1 min), 2 × (95°C, 1 min; 66°C, 1 min; 72°C, 1 min), 2 × (95°C, 1 min; 64°C, 1 min; 72°C, 1 min), 2 × (95°C, 1 min; 62°C, 1 min; 72°C, 1 min), 32–40 × (95°C, 1 min; 60°C, 1 min; 72°C, 1 min), and 72°C, 7 min. The PCR products were analyzed by electrophoresis in a 1.2% agarose gel and visualized by ethidium bromide staining. The following primers were used for semiquantitative RT-PCR: mouse Tspy (5′-CCT GAC TCC ACC TGG ACT GCT TAT AT-3′, 5′-TCA TCT TGG TTG CTG ATG ATG GAC GA-3′); β-actin (5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′, 5′-CCA CGT CAC ACT TCA TGA TGG A-3′), and Cre primers, as described above.

Histology and Immunohistochemistry

Whole embryos or dissected tissue were initially observed under the Leica MZ-FLIII fluorescence stereomicroscope and recorded with a DC300F digital imaging system. They were then fixed overnight in 10% formaldehyde-PBS (phosphate-buffered saline), pH 7.2 (Fisher Scientific, Hampton, NH) at 4°C. The tissues were incubated progressively through 10–30% sucrose in 2% DMSO / 100 mM PB (pH 7.2) for 12 h at each step at 4°C. They were then incubated in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC) at 4°C for 1 h prior to being embedded in the Tissue Freezing Medium over liquid nitrogen. Ten-μm cryosections were cut with a cryostat, air-dried for 30 min, and then washed 3 times in PBS for 5 min each. The sections were blocked with 3% bovine serum albumin (BSA) in PBS and then reacted with the primary antibodies at appropriate dilutions. After overnight incubation at 4°C, the slides were washed in PBS and then incubated with the appropriate secondary antibody for 3 h at 37°C. DNA was stained by the DAPI dyne in the mounting buffer (Vector Laboratories, Burlingame, CA). Fluorescent staining was examined with a Zeiss Axiophoto fluorescence microscope and recorded by a LEI-750 digital imaging system. Alexa488 conjugated rabbit anti-GFP antibody (1:400) and Alexa568 conjugated donkey antimouse IgG antibody (1:600) were obtained from Molecular Probes (Eugene, OR). Mouse anti-neurofilament M antibody (1:500), mouse anti-glial fibrillary acidic protein (GFAP) antibody (1:500), and mouse anti-neuronal nuclei (NeuN) antibody (1:150) were obtained from Chemicon International (Temecula, CA). Mouse anti-β-galactosidase antibody (1:100) was obtained from AbCam (Cambridge, UK).

For the immunohistological analysis of Cre expression, cryosections were air-dried and treated with a 3% H_2O_2 solution for 10 min. The sections were blocked with 10% normal goat serum in PBS and incubated with a rabbit anti-Cre antibody (1:1,000, Novagen, San Diego, CA) overnight at 4°C. The primary antibody was visualized by reacting with biotinylated antirabbit IgG antibody (1:600, Upstate Biochemicals, Lake Placid, NY) and horseradish peroxidase conjugated avidin (Vector Laboratories), using diaminobenzine as substrate. Nuclei were visualized by counterstaining with hematoxylin. The immunostained sections were examined and recorded with a Zeiss Axiophot microscope as before.

Analysis of TSPY Expression in Normal Human Testis

Antihuman TSPY mouse monoclonal antibodies were produced with the hybridoma technology against the full-length recombinant human TSPY by a commercial vendor (Vancouver Biotechnology, Vancouver, BC). The locations of epitopes were assigned by Western blotting of protein extracts from COS7 cells transfected with expression vectors consisting of pCS2plus (Rupp *et al.*, 1994; Turner and Weintraub, 1994) and cDNA fragments coding for 1–150 aa, 151–308 aa, or 1–308 aa of the

human TSPY (Zhang *et al.*, 1992). Western blotting was performed as described previously (Oh *et al.*, 2005).

Human testis sections were purchased from Ambion (LandMark Normal Tissue MicroArray, Austin, TX). The donors' age ranged from 63–78 years old. Sections were dewaxed and rehydrated through descending grades of alcohol to distilled water. The sections were treated with an antigen retrieval procedure in 100 mM Tris-HCl (pH 10) at 95°C for 30 min, incubated in 3% hydrogen peroxide, and washed in PBS. Nonspecific protein binding was blocked by pretreatment with 3% BSA in PBS. Sections were then incubated overnight in a humid chamber at 4°C with the respective TSPY monoclonal antibody (1:500 for clone #2, 1:3,000 for clone #7). The primary antibody was visualized as described above. The human study was performed under an exempt protocol approved by the Institutional Committee on Human Research, VA Medical Center, San Francisco.

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TSPY potentiates cell proliferation and tumorigenesis by promoting cell cycle progression in HeLa and NIH3T3 cells

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Abstract

Background: TSPY is a repeated gene mapped to the critical region harboring the gonadoblastoma locus on the Y chromosome (GBY), the only oncogenic locus on this male-specific chromosome. Elevated levels of TSPY have been observed in gonadoblastoma specimens and a variety of other tumor tissues, including testicular germ cell tumors, prostate cancer, melanoma, and liver cancer. TSPY contains a SET/NAP domain that is present in a family of cyclin B and/or histone binding proteins represented by the oncoprotein SET and the nucleosome assembly protein 1 (NAP1), involved in cell cycle regulation and replication.

Methods: To determine a possible cellular function for TSPY, we manipulated the TSPY expression in HeLa and NIH3T3 cells using the Tet-off system. Cell proliferation, colony formation assays and tumor growth in nude mice were utilized to determine the TSPY effects on cell growth and tumorigenesis. Cell cycle analysis and cell synchronization techniques were used to determine cell cycle profiles. Microarray and RT-PCR were used to investigate gene expression in TSPY expressing cells.

Results: Our findings suggest that TSPY expression increases cell proliferation *in vitro* and tumorigenesis *in vivo*. Ectopic expression of TSPY results in a smaller population of the host cells in the G_2/M phase of the cell cycle. Using cell synchronization techniques, we show that TSPY is capable of mediating a rapid transition of the cells through the G_2/M phase. Microarray analysis demonstrates that numerous genes involved in the cell cycle and apoptosis are affected by TSPY expression in the HeLa cells.

Conclusion: These data, taken together, have provided important insights on the probable functions of TSPY in cell cycle progression, cell proliferation, and tumorigenesis.

Background

The testis specific protein Y-encoded (TSPY) gene was one of the early genes to be identified from the human Y chromosome [1,2]. TSPY is embedded in a 20.4-kb DNA fragment that is tandemly repeated ~35 times in humans [3]. The 2.8-kb TSPY transcriptional unit consists of six exons and 5 introns distributed primarily on the short arm of the Y chromosome [2,4]. The bovine Y chromosome contains 50–200 copies of TSPY, while the rat Y chromosome contains a single copy. The mouse possesses a nonfunctional Tspy gene, on its Y chromosome, that harbors several stop codons within its open reading frame [5-7]. The human TSPY is expressed in both fetal and adult testes [2,4,8]. It is localized in the cytoplasm and nucleus of embryonic gonocytes and adult spermatogonial cells [4,8]. In particular, the spermatogonial cells are the only cells in the male capable of entering both mitotic and meiotic cell division. The exact function of the TSPY gene product is thus far unknown. It has been hypothesized to regulate the normal proliferation of spermatogonia and marks the entry of the spermatogonia into the meiotic differentiation [9].

TSPY is expressed in adult testis as a phosphoprotein with an apparent molecular weight of 38 kD [4]. It harbors a SET/NAP domain, conserved among members of a protein family, represented by the SET oncoprotein and nucleosome assembly protein-1 (NAP-1) respectively. Major members of this protein family include SET, NAP-1, TSPY, differentially expressed nucleolar TGF-β1 target (DENTT) [10,11]/cell division autoantigen-1 (CDA1) [12]/TSPX [13]. SET was initially identified in a patient with acute undifferentiated leukemia, who harbored an intrachromosomal translocation on chromosome 9 [14-16] and demonstrated to bind B-type cyclins [17]. SET regulates the G_2/M transition by modulating cyclin B-cyclin-dependent kinase 1 (CDK1) activity [18]. NAP-1 interacts with B-type cyclins in budding yeast and frogs [17]. In *Saccharomyces cerevisiae*, cells that lack NAP-1, the Clb2 (B-type cyclin) was unable to efficiently induce mitotic events [19,20]. Over-expression of SET or CDA1 results in an inhibitory effect on cell cycle progression at the G_2/M phase [18], suggesting that SET/NAP-containing proteins are cell cycle regulators.

Deletion mapping for the gonadoblastoma locus on the Y chromosome (GBY) [21] has localized this oncogenic locus in a critical region (~1–2 Mb) on the short arm of this chromosome that contains most of the functional copies of the TSPY gene [22,23]. Elevated levels of TSPY protein have been observed in gonadoblastoma, thereby providing supporting evidence for TSPY as a likely candidate for the GBY [4,9,24,25]. TSPY is also expressed in testicular carcinoma-in-situ (CIS) [4,25], seminomas [24], prostate cancer specimens/cell lines [26-28], melanomas [29] and hepatocellular carcinoma [30]. To test the hypothesis that TSPY is involved in cell cycle regulation and its aberrant expression could contribute to the overall tumorigenesis, we have examined the effects of ectopic expression of TSPY in cell proliferation and tumorigenesis in athymic nude mice, using the tetracycline (Tet-off) regulation system in human HeLa and mouse NIH3T3 cells [31]. Our results suggest that ectopic expression of TSPY increases cell proliferation *in vitro* and tumorigenesis *in vivo*. Expression of TSPY expedites the transition of the cells through the G_2/M phase of the cell cycle, indirectly up-regulates pro-growth genes and down-regulates apoptosis inducing molecules and growth inhibitory genes, thereby promoting cell proliferation in both cell cultures and whole animals.

Methods

Plasmids and stable cell transfection

The TSPY cDNA [2] was inserted at the EcoR1 site of the bicistronic vector, pTRE-IRES-GFP (designated as pTIG). The resulting construct (pTIG-TSPY) is capable of expressing both TSPY and EGFP under control of a modular tetracycline-responsive promoter. HeLa and NIH3T3 Tet-off cells harboring a stably integrated tetracycline transactivator gene were purchased from Clontech-BD BioSciences (Mountain View, CA). They were cultured in DMEM media containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 400 μg/ml G418 (Invitrogen-Life Technologies, Carlsbad, CA) at 37°C in 5% CO_2.

To generate stable cell lines conditionally expressing TSPY, the Tet-off cells were co-transfected with the pTIG-TSPY or pTIG vector and the hygromycin resistance marker, pTK-Hyg, at a ratio of 20:1, using either the Lipofectamine Plus (Invitrogen-Life Technologies, Carlsbad, CA) or FuGene6 (Roche, Alameda, CA) reagents, according to the instructions of the respective manufacturers. The cells were selected with the complete medium plus 300 μg/ml hygromycin (Invitrogen-Life Technologies, Carlsbad, CA) at a density of 4×10^5 cells per 100 mm culture dish. Cell colonies were selected with or without 2 ng/ml doxycycline (Dox) (Sigma, St. Louis, MO) in the selective media for 2–3 weeks. Positive colonies were isolated individually and clonally expanded as sub-lines. Alternatively, the colonies were pooled and isolated by preparative flow cytometry based on their EGFP expression using a FACSVantage SE Cell Sorter (Becton Dickinson, Frankilin Lakes, NJ) at the Laboratory for Cell Analysis, Cancer Center, University of California, San Francisco. Cells expressing EGFP were collected on ice in fresh media and immediately re-plated in culture dishes with complete media. Cells isolated with either strategy were then analyzed for their responsiveness to doxycycline regulation of their TSPY and EGFP transgenes using

western blotting and fluorescence microscopy respectively.

Immunofluorescence and western blotting

EGFP expression in cultured cells was observed directly under a Zeiss Axiophot fluorescence microscope using an excitation filter HQ 480/40 and an emission filter HQ 510 LP (Chroma Technology Corp., Rockingham, VT). TSPY expression was detected by indirect immunofluorescence according to established procedures. The cells were stained with a polyclonal TSPY antiserum [9] at 1:100 dilution at 4°C overnight, rinsed 3 times with PBS, incubated with a goat anti-rabbit IgG antibody conjugated to Texas Red (1:1000 dilution) for 30 minutes at room temperature, and analyzed with the Zeiss fluorescence microscope and appropriate filter set for Texas Red.

Western blotting of total cell lysates from HeLa or NIH3T3 Tet-off cells grown in the presence or absence of 2 ng/ml Dox was processed according to established procedures, using TSPY antisera and monoclonal antibodies, as described previously[32]. Immunoblot signals were detected by enhanced chemiluminescence (ECL) technique (Amersham, Piscataway, NJ).

Cell proliferation analysis

Cell proliferation was analyzed by XTT assay, based on the cleavage of the tetrazolium salt XTT in the presence of an electron-coupling reagent by the succinate-tetrazolium reductase whose activity was directly associated with number of viable cells. HeLa or NIH3T3 Tet-off cells stably transfected with pTIG-TSPY or pTIG were grown on 100 mm dishes in culture media in the presence or absence of 2 ng/ml doxycycline for 3 days. Cells were then seeded at a concentration of 5×10^3 cells/well in 100 μl culture media with or without Dox on a 96-well microtiter plate, incubated at 37°C with 5% CO_2 and analyzed in triplicates at 24 h intervals for 4 days. Fifty μl XTT labeling mixture was added to each well 4 hours before each spectrometric measurement.

Colony formation assay

HeLa and NIH3T3 Tet-off cells were transfected with either pTIG-TSPY or pTIG plasmid and cultured for 3 weeks in a selective medium containing 300 μg/ml hygromycin and with or without 2 ng/ml Dox. The resulting colonies were stained with Giemsa and counted manually. Each 100 mm dish represented 1:10 dilution of cells from a single well, which had been transfected with 1 μg of TIG-TSPY or vector and 0.05 μg pTK-Hyg selectable marker.

Tumorigenicity assay in athymic nude mice

Female 8-week old athymic nu/nu mice (Charles River Laboratories, Wilmington, MA) were used for tumorigenicity assays. Subconfluent and exponentially growing monolayer cells of each cell line were trypsinized, washed and resuspended in PBS. One million TSPY-transfected HeLa cells or 10 millions of similarly transfected NIH3T3 cells in 100 μl PBS were inoculated subcutaneously into the flanks of the nude mice. Control cells, transfected with pTIG or non-transfected parental cells, were inoculated similarly. Six animals were used for inoculation of each cell type. The mice were fed with drinking water with or without 2 μg/ml of doxycycline. Tumor growth was measured by the tumor volume, in mm^3, estimated from the length (L) and width (W) of the tumors and the formula $(L \times W^2)/2$ [33]. The tumorigenicity assays were terminated by sacrificing the mice at 5 weeks (for HeLa cells) or 7 weeks (for NIT3T3 cells) after inoculation. All animal studies were conducted under an approved protocol by the Institutional Animal Care and Use Committee of the VA Medical Center, San Francisco.

Cell synchronization and cell cycle analysis

Equal number of HeLa Tet-off cells stably transfected with either pTIG-TSPY or pTIG were grown overnight, washed with PBS, and fed with fresh media containing 2.5 mM thymidine (for G_1/S synchronization) or 80 ng/ml colcemid (for M phase synchronization) [34,35]. Cells were synchronized in the respective media for 24 hours, washed 3 times with PBS and released into fresh complete media. At specific time points, the media was removed and the cells were washed in PBS, trypsinized, collected, washed again in PBS, placed in 1–3 ml of ice cold 70% ethanol and incubated at -20°C for 1 hour – overnight. Cells were then incubated in 10 μg/ml propidium iodide/0.1% Triton X-100/0.1% RNase in PBS solution at 37°C for 30 minutes in the dark. They were resuspended in 0.5 ml PBS and analyzed with a FACSCalibur flow cytometer at the Laboratory for Cell Analysis, UCSF Cancer Center. Cell cycle analysis was performed using ModFit LT (Verity Software House, Topsham, ME), FlowJo (Tree Star, Ashland, OR), and Openlab (Improvision, Lexington, MA) software.

Microarray analysis of gene expression profiles

Total RNA was isolated from respective cell populations with TRIzol reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy Mini Kit (Qiagen, Valencia, CA) in accordance with the manufacturer's instructions. The quality of RNA was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Five μg of RNA was converted into double-stranded cDNA using a cDNA synthesis kit (Affymetrix, Santa Clara, CA) with a special oligo$(dT)_{24}$ primer containing a T7 RNA promoter site added 3' to the poly(T) tract. Biotinylated cRNAs were generated from cDNAs using the bioarray high yield RNA transcript labeling kit (Enzo Life Science, Farmingdale, NY) and subsequently purified with the RNeasy kit (Qia-

gen, Valencia, CA). Complementary RNA probe derived from each sample was fragmented and hybridized to GeneChip® Human Genome U133 Plus 2.0 Array containing 47,000 transcripts and variants using the Affymetrix GeneChip Fluidics Station 450. Arrays were scanned by an Affymetrix Gene Scanner 3000, the image files were processed using Microarray Analysis Suite version 5.0 (Affymetrix, Santa Clara, CA). Each biological sample was analyzed with three technical replicates. All the 54,675 gene spots were filtered based on spot quality, statistical test and corrections before subsequent analyses. To identify differentially expressed transcripts, all spots were first normalized by scaling total chip fluorescence intensity to a common value of 100 prior to comparison, and a normalization value was set at 1. To minimize the potential false-positives using t-test alone, the t-test p-value of each gene when performing a statistical test was corrected by multiple testing corrections, which adjust the individual p-value for each gene to keep the overall error rate (or false positive rate) to less than or equal to a cutoff at $p < 0.005$. Multiple testing corrections based on Bonferroni step-down method were used. The corrected p-value is calculated and considered to be significant if it is less than 0.05. The false discovery rate applied in the tests was 5%. Data and statistical analyses were performed with Genespring 7.0 software (Silicon Genetics, Redwood City, CA).

Semi-quantitative RT-PCR was used to validate genes differentially expressed from data obtained with microarray analysis, as described previously [27]. The primers were derived from the corresponding cDNA sequences capable of amplifying roughly 100 to120-bp fragments from transcripts of the respective genes (Table 1). GADPH was included as a control in all the RT-PCR experiments. The number of cycle for each sample was independently determined to insure the reaction was terminated during the exponential phase of the amplification. PCR reaction products were separated on 1.5% agarose gels using ethidium bromide for visualization. The relative abundance of each PCR product was determined by quantitative analysis of digital photographs of the gels viewed under UV light (LabWorks software; UVP, Inc.).

Results

Tet-off regulation of TSPY gene expression

TSPY has been hypothesized to play a role(s) in regulation of germ cell proliferation and meiotic division. When it is ectopically expressed in cells incompatible with germ cell proliferation and/or division, it exerts a yet-to-be defined oncogenic effect(s) and contributes to the overall tumorigenic process(es) in susceptible cells of the affected organ/tissue [9,24,28]. To evaluate the effects of such ectopic TSPY expression, we had used the Tet-off transgene regulation system to manipulate its expression in HeLa and NIH3T3 cells [31]. HeLa cells are human female cervical carcinoma cells without the Y chromosome (and hence TSPY genes) while the NIH3T3 cells are normal (non-tumorigenic) mouse fibroblastic cells. Using the bicistronic vector, TIG, we were able to demonstrate that both TSPY and EGFP could be co-expressed in the same stably transfected cells (Figure 1A, B) and could be manipulated with the addition of doxycycline in the culture media (Figure 1C, D). EGFP was distributed relatively evenly among different compartments of most cells, while TSPY was located primarily in the cytoplasm and lightly in the nuclei. A small portion of cells, however, showed substantial nuclear localization or aggregated distribution of the TSPY protein (Figure 1F, H, arrows). Similar nuclear location of TSPY had been observed in various tumor specimens [9,25]. A recent report suggests that a CD2-dependent phosphorylation of the tyrosine reside at position 300 of the predominant TSPY isoform is essential for

Table 1: Gene-specific primers used in semi-quantitative RT-PCR analysis

Gene	Primers	Sequences
RGC32	Forward	GAGCGCCACTTCCACTACG
	Reverse	AGTGAATCTGCACTCTCCGAG
PDGFC	Forward	ACTCAGGCGGAATCCAACC
	Reverse	CTTGGGCTGTGAATACTTCCATT
WNT5A	Forward	TTGGTGGTCGCTAGGTATGAA
	Reverse	AGTGGCACAGTTTCTTCTGTC
CCND2	Forward	AGGCGGTGCTCCTCAATAG
	Reverse	TATCCCGCACGTCTGTAGGG
CUL1	Forward	ACCAGTCAAACCAAGCACGAG
	Reverse	GTCTGCCCCTTTTTCGACTTAG
IGFBP3	Forward	AGAGCACAGATACCCAGAACT
	Reverse	TGAGGAACTTCAGGTGATTCAGT
TIMP1	Forward	CTTCTGCAATTCCGACCTCGT
	Reverse	CCCTAAGGCTTGGAACCCTTT
SPARC	Forward	AGCACCCCATTGACGGGTA
	Reverse	GGTCACAGGTCTCGAAAAAGC

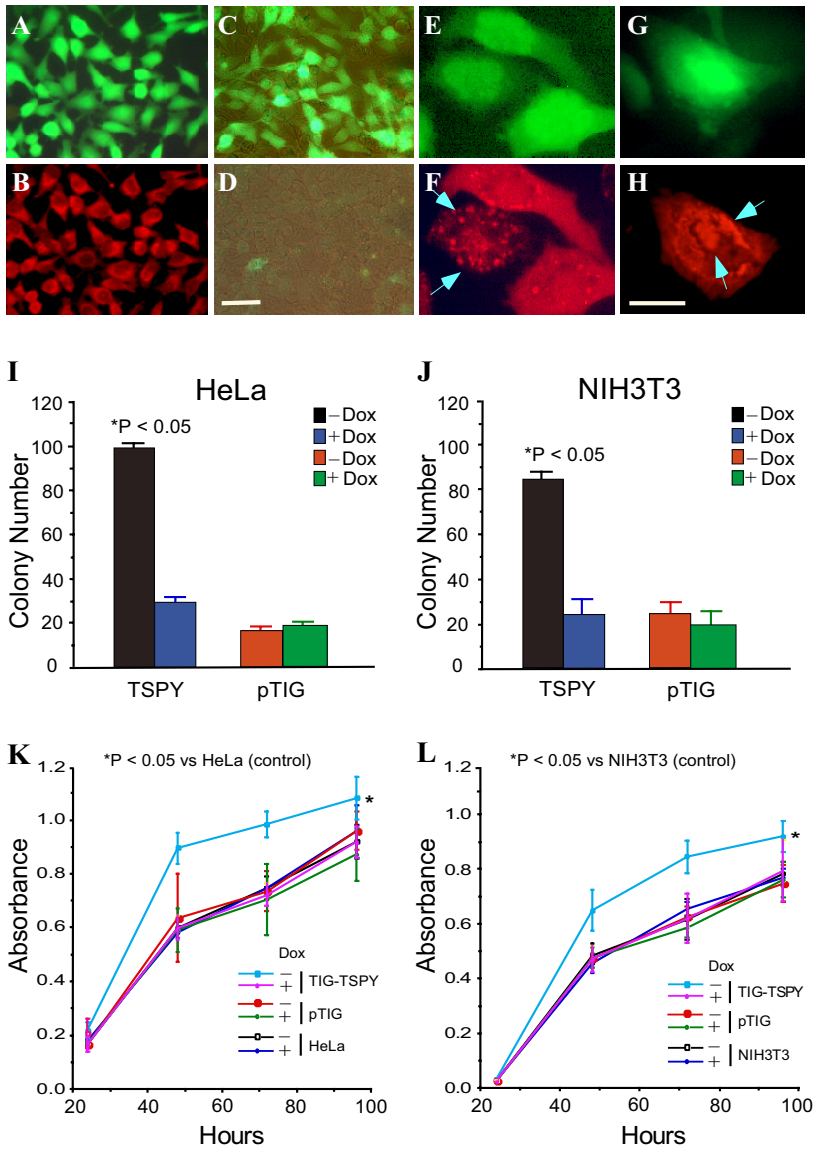

Figure 1

A-H) Tet-off regulation of TSPY and EGFP expression in HeLa cells. A bicistronic transgene capable of expressing both TSPY and EGFP in the same transcriptional unit was stably transfected to HeLa Tet-off cells. EGFP fluorescence (A) and TSPY (B) were detected by direct observation and immunofluorescence respectively. Such expression (i.e. EGFP in C) could be suppressed by administration of doxycycline in the culture media (D). EGFP was located in both cytoplasm and nuclei of the host cells (E, G) while TSPY could display scattered locations along the periphery and within the nuclei, in addition to its cytoplasmic distribution (F, H, blue arrows). I-L) Effects of TSPY expression in cell transfection efficiency and proliferation in HeLa and NIH3T3 Tet-off cells. HeLa (I) and NIH3T3 (J) Tet-off cells were co-transfected with either TIG-TSPY (TSPY) construct or the vector pTIG alone with the TK-Hyg plasmid and were selected in culture media with hygromycin with (+) or without (-) doxycyclinc (Dox). Both HeLa (I) and NIH3T3 (J) cells expressing TSPY (i.e. TSPY without doxycycline) consistently formed higher numbers of colonies than those repressing the same transgene (i.e. TSPY with doxycycline) while those expressing EGFP in the vector (pTIG) alone did not show any differences in the transfection efficiency in the absence or presence of doxycycline. Cell proliferation assays demonstrated that HeLa (K) and NIH3T3 (L) Tet-off cells over-expressing TSPY (without doxycycline) proliferated at a faster rate(s) than those repressing the TSPY gene (with doxycycline). Again, cells harboring the vector (pTIG) alone did not show any difference in their proliferative activities in media with or without doxycycline. Bars in D and H represent 40 μM in A-D and 10 μM in E-H respectively. * Student's t-test analysis indicated that there were statistical significance in differences between TSPY-expressing cells and vector-alone cells.

its nuclear translocation [36]. Currently, the significance of such differential distribution of TSPY in the cell compartments is uncertain. The present results demonstrate that the TSPY and EGFP are expressed abundantly and manipulated effectively with the Tet-off system in stably transfected HeLa and NIH3T3 cells.

Over-expression of TSPY increases the colony formation efficiency and cell proliferation

To determine the effects of ectopic TSPY expression in cell growth, the transfection efficiency of the pTK-Hyg marker was determined in combination with either the TIG-TSPY construct or TIG vector in HeLa and NIH3T3 Tet-off cells. Approximately 3-fold higher numbers of colonies were observed in TIG-TSPY transfected cells selected without the doxycycline, i.e. over-expressing TSPY and EGFP, over identically transfected cells selected in media containing doxycycline, i.e. repressing the TSPY and EGFP expression (Figure 1I, J, left panel). Such differential efficiency of colony formation was absent in cells transfected with the TIG vector alone either with or without doxycycline in the selection media (Figure 1I, J, right panel). Although we cannot rule out completely that co-expression of TSPY might enhance the TK-Hyg gene expression, these results, and those from cell proliferation analysis described below, suggested that over-expression of TSPY enhances the efficiency of cell growth under such selection.

To evaluate the effects of TSPY expression in cell proliferation, TIG-TSPY, TIG transfected cells and the respective parental cells were analyzed with the XTT cell proliferation assay. Both HeLa and NIH3T3 Tet-off cells over-expressing TSPY-EGFP showed consistently 30–45% higher proliferative activities than those whose transgenes were repressed by doxycycline. Cells transfected with the vector alone, similar to the parental cells, did not show any proliferative differences in the presence or absence of doxycycline in the media (Figure 1K and 1L). These findings suggest that ectopic expression of TSPY potentiates cell proliferation in cultured cells.

TSPY expression accelerates tumor growth in nude mice

Tumorigenicity assay was used to correlate the proliferative effects of TSPY in cultured cells to those in whole animals. HeLa Tet-off cells transfected with either TIG-TSPY or TIG vector alone and parental cells were inoculated subcutaneously on the flanks of female athymic nude mice. The mice were fed with drinking water either with or without doxycycline for 5 weeks. The size of tumors formed at the inoculation site was measured with standard procedure at the respective time points (Figure 2A, B). An accelerated tumor growth was observed in mice inoculated with HeLa Tet-off cells over-expressing TSPY (without doxycycline in their drinking water) compared to the group repressing TSPY (with doxycycline in their drinking water). No accelerated tumor growth was observed in the parental or the groups inoculated with HeLa cells harboring the vector alone, with or without doxycycline in their drinking water. These results suggested that ectopic expression of TSPY increased tumor growth in athymic nude mice.

NIH3T3 cells are established mouse fibroblasts that normally do not form tumors in athymic animals. Using a similar tumorigenicity assay, small size tumors were observed in 5 out of 6 mice inoculated each with about 10 millions cells harboring and expressing TIG-TSPY (without doxycycline in drinking water), while no tumors were observed in the group of animals inoculated with similar number of transfected cells, but fed with doxycycline in their drinking water (Figure 2D, E). Again, mice inoculated with the NIH3T3 cells transfected with vector alone or the parental cells did not show any tumor formation, with or without doxycycline in their drinking water. The expression of the TSPY-EGFP transgene in the tumors of these nude mice, fed with normal drinking water, could be confirmed by direct observation of EGFP fluorescence in the whole animals (Figure 2C, F).

TSPY expedites a rapid transition of G_2/M of the cell cycle

Members of the SET/NAP family of proteins had been demonstrated to bind type B cyclins and exert biological effects on cell cycle progression. Our study, so far, showed that ectopic expression of TSPY in HeLa and NIH3T3 cells potentiated cell proliferation in vitro and tumor growth in nude mice. To evaluate the likely cell cycle stage(s) in which TSPY affected its pro-growth function, both exponentially growing and synchronized HeLa Tet-off cells harboring either the TIG-TSPY transgene or TIG vector alone were analyzed with flow cytometry techniques. Exponentially growing HeLa Tet-off cells harboring the vector alone showed similar patterns in their cell cycle distribution when they were grown in the presence or absence of doxycycline (Figure 3A) while those over-expressing TSPY showed a small portion of its cells in the G_2/M phase (Figure 3B, red line). When expression of the TSPY transgene was repressed with doxycycline in the media (Figure 3B, blue line), the distribution of cells among the different stages of the cell cycle was similar to those of control cells (Figure 3A).

To determine why there was fewer number of TSPY expressing HeLa Tet-off cells at G_2/M phase, as compared to HeLa Tet-off harboring the vector alone, the cells were examined further with cell synchronization and cell cycle analysis with flow cytometry. Cells were synchronized at the G_1/S boundary, released into the S phase and analyzed with flow cytometry thereafter at 0, 6, 12, 24, 36 and 48 hours. The relative percents of the cells distributed at different stages (G1, S and G_2/M) of the cell cycle were deter-

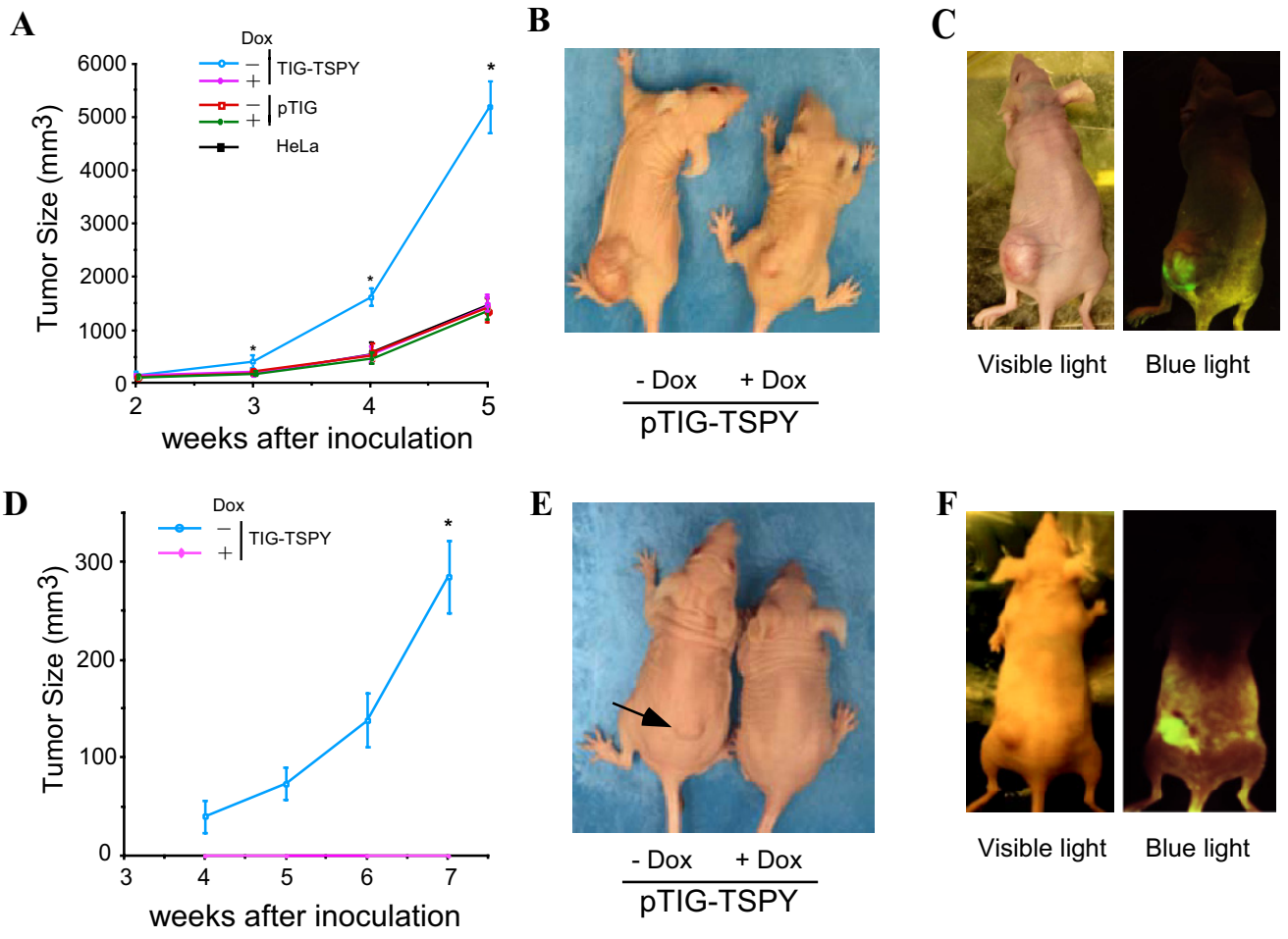

Figure 2

TSPY enhances tumor growth in athymic nude mice. Tumorigenicity assays demonstrated that HeLa Tet-off cells (A) over-expressing TSPY (- Dox) formed tumor at a faster rate in athymic nude mice than those whose TSPY expression was repressed with doxycycline (+ Dox) in the drinking water. Cells harboring the vector alone (pTIG) with or without doxycycline administration or the parental cells (HeLa) showed similar tumor growth rates as that of cells repressing TSPY transgene (+Dox) in the host animals. NIH3T3 Tet-off cells are non-tumorigenic cells that normally do not form tumor in athymic hosts. Inoculation of these cells over-expressing TSPY (D, – Dox), however, produced small size tumors in 5 of 6 nude mice (E) while no tumor was observed in animals inoculated with the same cells and fed with doxycycline-containing water (+Dox). Inoculation of NIH3T3 Tet-off cells harboring the vector alone or parental cells did not produce any tumor in the same hosts fed with drinking water with or without doxycycline (data not shown). B and E show examples of athymic nude mice from respective inoculations at the end of the experiments. Co-expression of EGFP provided a convenient means to observe directly the tumor growth (size) in nude mice (C, F). * Student's t-test analysis indicated that there were statistical significance in differences between TSPY-expressing cells and vector-alone cells.

mined from the respective flow cytometry charts. At 6 hours, most cells in both populations were mostly at S phase while at 12 hours, some cells had gone through S and G_2/M and reached G1. However, the majority of the cells over-expressing TSPY reached the second S phase again in 24 hours (Figure 3D) while cells harboring just the vector alone transited the cell cycle stages more slowly and had only ~35% of cells reaching the second S phase (Figure 3C). The synchronization effects seemed to dissipate at 36 and 48 hours in both populations (Figure 3C, D) and the distributions of their cells in various cell cycle stages resembled those of exponentially growing cells (as in Figure 3A, B respectively). Since cells over-expressing TSPY showed a smaller number in G_2/M, such rapid transition of the cell cycle after the G_1/S phase synchronization suggested that the cell could progress through the G_2/M phase faster than those harboring the vector alone. To confirm our postulation that TSPY mediated a fast transit

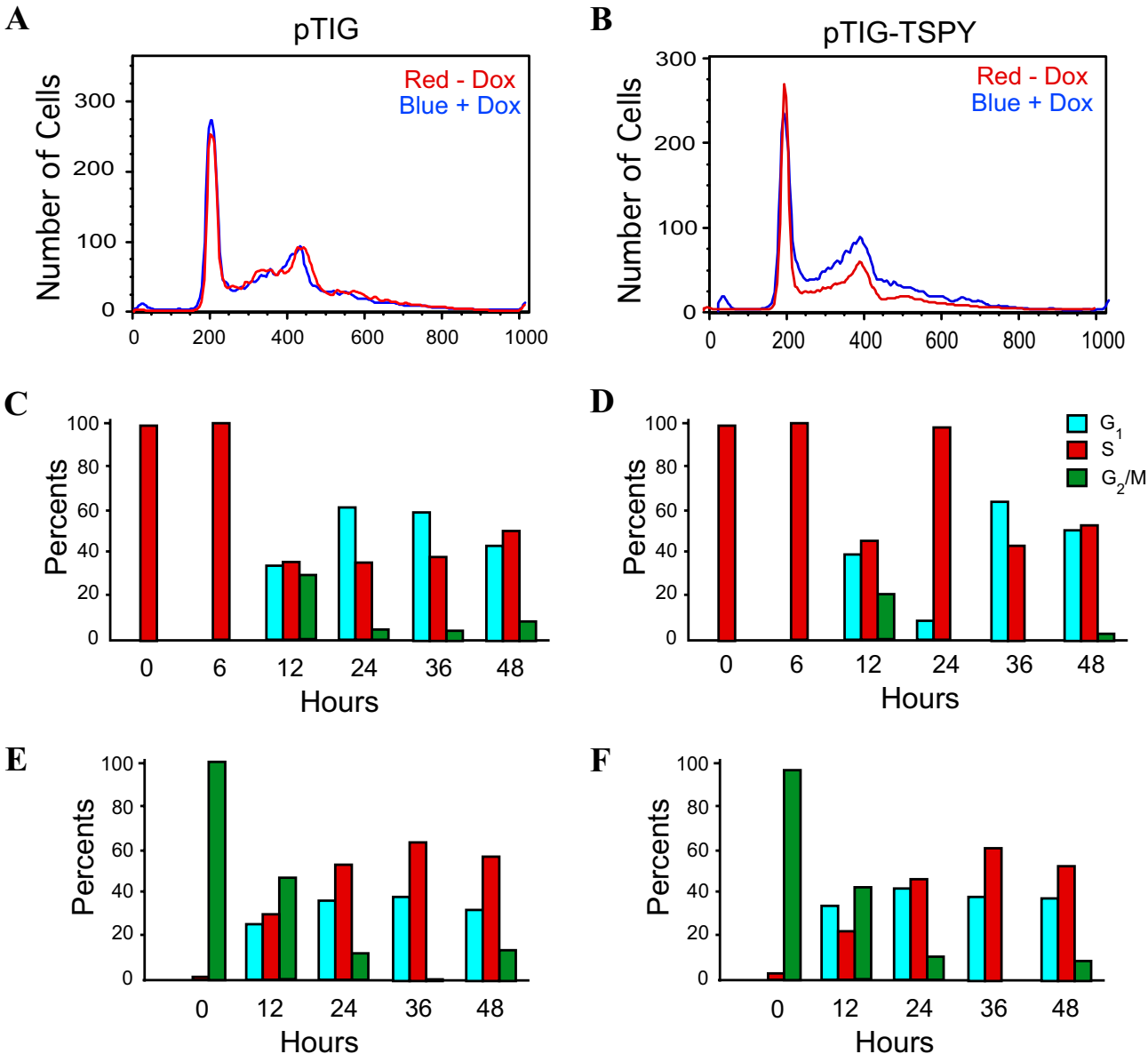

Figure 3

TSPY accelerates G_2/M transition in cell cycle. Flow cytometry analysis of HeLa Tet-off cells harboring either the vector, pTIG, (A) or pTIG-TSPY (B) construct showed a reduced number of cells at G_2/M phase in cells over-expressing TSPY (B, -Dox) than those repressing TSPY (B, +Dox) or harboring the vector alone (A, +/- Dox). To evaluate the probable reason(s) for such differential distribution of cells in various phases of the cell cycle, HeLa Tet-off cells harboring either the vector alone (C) or pTIG-TSPY construct (D) were synchronized at G_1/S, released into S phase and analyzed similarly with flow cytometry at 0, 6, 12, 24, 36 and 48 hours after the release. HeLa cells harboring pTIG-TSPY construct (D) progressed at a faster rate than those harboring the vector alone (C), and reached in the second S phase within 24 hours after release from the synchronization while significant number of cells harboring the vector alone (C) remained in G_1 phase at the same time. Such synchronized cell cycle progression disappeared towards 36 and 48 hours in both cell populations (C, D). When the cells were synchronized at the G_2/M phase, released into mitosis, and analyzed similarly with flow cytometry, such accelerated rate of cell cycle progression were not observed readily between populations harboring the vector alone (E) and TSPY (F). Again, cells over-expressing TSPY showed a reduced number(s) at G_2/M (e.g. 24 and 36 hour time points in D and all time points in F as compared to those in C and E respectively) when they were over-expressing TSPY, resembling those of asynchronous cells (B).

through G_2/M, both HeLa cell populations were synchronized at metaphase by colcemid treatment, released into cell cycle and analyzed thereafter at 0, 12, 24, 36, and 48 hours with flow cytometry. Our results showed that cells expressing TSPY progressed through the G_1 and S phases (Figure 3F) at similar rates to those lacking TSPY expression (Figure 3E). Again, at 36 and 48 hours after their release, cells in both populations (i.e. harboring TIG-TSPT and TIG) showed a similar cell cycle stage distribution, resembling those of exponentially growing and asynchronous cells. We surmise that the facilitating function of TSPY in the cell cycle was less obvious because the synchronization was at metaphase, immediate beyond the G_2 stage in which TSPY is postulated to be effective in expediting cell cycle progression. Nevertheless, one could still observe fewer percents of cells were at G_2/M in cells over-expressing than those lacking TSPY at these time points, thereby supporting a role of TSPY at this stage of the cell cycle, as discussed above.

Cyclin B1 is a mitotic cyclin whose expression is primarily at late S and G_2 [37,38]. Its degradation by the anaphase promoting complex is essential for the cell to exit mitosis [39-42]. Hence, the amount of cyclin B1 varies with the different stages of the cell cycle, i.e. the lowest at G_1 and highest at G_2/M. To substantiate the effects of TSPY in cell cycle progression, we had examined the relative amount of cyclin B1 in HeLa Tet-off cells harboring either TIG-TSPY or TIG vector alone, after they were synchronized at G_1/S and released into the cell cycle. Cells were harvested at 2 hour-intervals, starting at 0 hours and ending at 22 hours. Total protein extracts were prepared from these cells and processed for western blotting with cyclin B1, tubulin and TSPY antibodies. Both cell populations showed minimal level of cyclin B1 at 0 hours immediately after release from G_1/S phase synchronization. The levels of cyclin B1 in HeLa cells harboring the vector alone increased gradually starting at 6 hours and peaked at 10 hours and gradually declined through detectable levels to 14–16 hours (Figure 4A). Cells expressing TSPY showed a detectable level immediately after release from G_1/S synchronization (Figure 4B). The level increased gradually and peaked at 10 hours, but fell rapidly, although still detectable, thereafter at 12–14 hours. Repeat probing of the same/similar filters with a tubulin antibody showed that the loading was relatively even among the various samples (Figure 4A, lower panel, 4B, middle panel). Cell harboring the TIG-TSPY transgene expressed TSPY at consistent level throughout the cell cycle (Figure 4B, lower panel). These findings suggested that TSPY expressing cells exited the G_2/M phase faster than those lacking such expression.

Differential expression of growth-related genes in HeLa cells over-expressing TSPY

To determine how TSPY expression affects cell growth and cell cycle progression, microarray analysis was performed on HeLa Tet-off cells expressing TIG-TSPY and compared with those harboring the TIG vector alone, using the Affymetrix GeneChip containing 47,000 human transcripts and variants. Our results showed that TSPY and a limited number of genes were differentially expressed between the two cell populations. TSPY was expressed at the highest level while others were consistently affected at modest levels. The present findings were consistent with the fact that the two HeLa cell populations were almost identical, except the high TSPY expression in one and not the other. To translate sets of differentially regulated genes at each stage into functional profiles, 181 differentially expressed genes were analyzed with the web-based program, Gene Ontology Tree Machine (GOTM) [43]. Statistical analysis was performed to identify the most important Gene Ontology categories for the input gene sets and to suggest their potential biological importance in the categories. Three biological processes, cell cycle, phosphate transport, and neuromuscular development, were preferentially represented among the differentially expressed genes. Both cell cycle and phosphate transport could be related to cell growth, and hence are relevant differences between TSPY over-expressing and vector alone cell populations. The exact nature of differential expression of genes involved in neuromuscular development is uncertain. However, previous transgenic mouse studies demonstrated that the human TSPY promoter could be preferentially active in neurons of pre- and postnatal brains, suggesting a possible function of this Y chromosome gene in neural development [32]. The various cell cycle related processes affected by the constitutive expression of TSPY are illustrated (Figure 5). Table 2 lists 25 cell cycle related genes whose expression was either up- or down-regulated by over-expression of TSPY. Among the up-regulated genes were several oncogenes (epidermal growth factor receptor (ERBB) and members of the WNT (WNT5A) and RAS families (RAP1A)), growth factors (PDGFC, EGF-related, ANKRD15, RGC32, NANOS1), cyclin D2 (CCND2), a co-factor for the hypoxia inducible factor 1A (EP300), an apoptosis inhibitor (GSPT1) and an antigen (CD24) highly expressed in small cell lung carcinoma. The down-regulated genes included an inhibitor for CDK4/CDK6 (CDKN2B), transforming growth factor β3, pro-apoptotic factors (CLU and IGFB3), and an inhibitor of MAP kinases (DUSP5). In particular, the CCND2 gene (encoding cyclin D2) resides on chromosome 12p that is frequently amplified and expressed at high levels in testicular germ cell tumors. Cyclin D2 complexes with CDK4 or CDK6 to mediate G_1/S transition and promote cell proliferation. Indeed CCND2 expression was up-regulated in HeLa Tet-off cells over-expressing TSPY. Con-

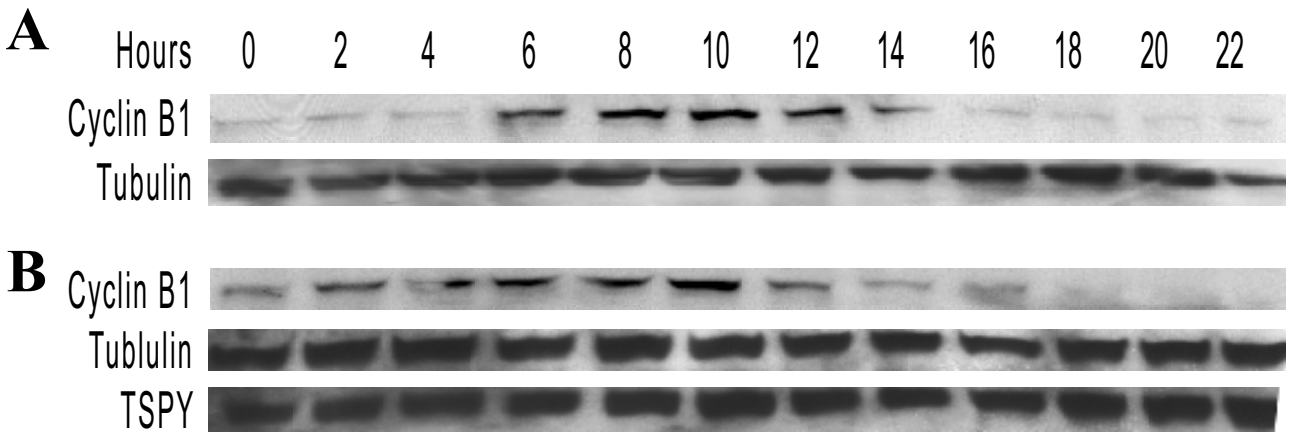

Figure 4
The rapid G_2/M transition of HeLa cells over-expressing TSPY is associated with an early degradation of the mitotic cyclin B1. HeLa Tet-off cells harboring the vector alone (A) and TIG-TSPY construct (B) were synchronized at G_1/S, released into S phase, harvested and analyzed with western blotting with various antibodies at 2-hour intervals for 22 hours. The mitotic cyclin B1 is synthesized normally in late S phase and G_2 and is rapidly degraded before the cell exits mitosis. In cells over-expressing EGFP in the vector alone, the levels of cyclin B1 increased at 6 hours after the G_1/S release, peaked at 10 hours and gradually decreased until 14–16 hours (A, top row) while in those over-expressing both EFGP and TSPY, the levels of cyclin B1 increased gradually at 2 hours after the G11/S release, peaked at 10 hours and rapidly reduced thereafter (B, top row). The filters were re-probed with tubulin antibody (A, bottom row; B, middle row) showing relatively even loading of the samples. TSPY was uniformly expressed and detected in samples of cells over-expressing this transgene (B, bottom row).

versely, an inhibitor, CDKN2B, against activation of CDK4/CDK6 was down-regulated in the same cells, thereby further supporting the possible role of TSPY in the CCND2-CDKN2B cell cycle regulation. In additional to CCND2, another genes, the transmembrane and tetratricopeptide repeats (TMTC1) from chromosome 12p was also up-regulated by the ectopic expression of TSPY in HeLa cells. Currently the function of TMTC1 is unknown. Other tetratricopeptide repeats-containing proteins, such as subunits cdc16, cdc23 and cdc27 of the anaphase promoting complex (APC), could play important roles in protein-protein interaction, cell division and receptor signaling [44-46].

To confirm the microarray data, semi-quantitative RT-PCR was performed on 4 of the up-regulated genes and 4 of the down-regulated genes from Table 2 (bold typed). Figure 6A showed the results of the RT-PCR from HeLa Tet-off cells harboring either pTIG or pTIG-TSPY. RGC32, PDGFC, WNT5A, and CCND2 were confirmed as up-regulated genes in cells over-expressing TSPY; each showed a slight increase in fold expression compared to the microarray data. CUL1, IGFBP3, TIMP1, and SPARC were confirmed as down-regulated genes in cells expressing TSPY (Figure 6B); each of these also showed a slight change in fold expression compared to the microarray data. These results confirmed that cells expressing TSPY could indirectly up-regulate genes involved in the cell growth and proliferation and down-regulate genes involved in the cell cycle inhibitors, growth suppressors, and pro-apoptotic factors.

Discussion

TSPY is a unique gene in the human genome. Although its transcriptional unit is only approximately 2.8-kb in size, it is embedded in a 20.4-kb tandemly repeated unit that shows >98% homology among the members of the TSPY gene family [47]. Numerous studies suggest that most of the repeat units are functional capable of coding for a variety of polymorphic TSPY proteins [28,48,49]. For one sequenced individual, the TSPY repeat units constitutes ~0.7 MB of DNA on the short arm and only one single copy on the long arm of the Y chromosome [48,50,51]. Currently, the exact nature of such tandem repetition of a functional gene and its flanking sequences is uncertain. However, we surmise that TSPY repeats could be hot spots for genetic rearrangements and/or transcriptional dysregulation, resulting in ectopic expression of TSPY variant transcripts and proteins in tissues that normally do not express this Y-located gene [9,29,30] and variation in copy numbers and/or genetic rearrangements [52].

TSPY is a member of a protein superfamily that is defined by a conserved 191 amino acid domain, designated as

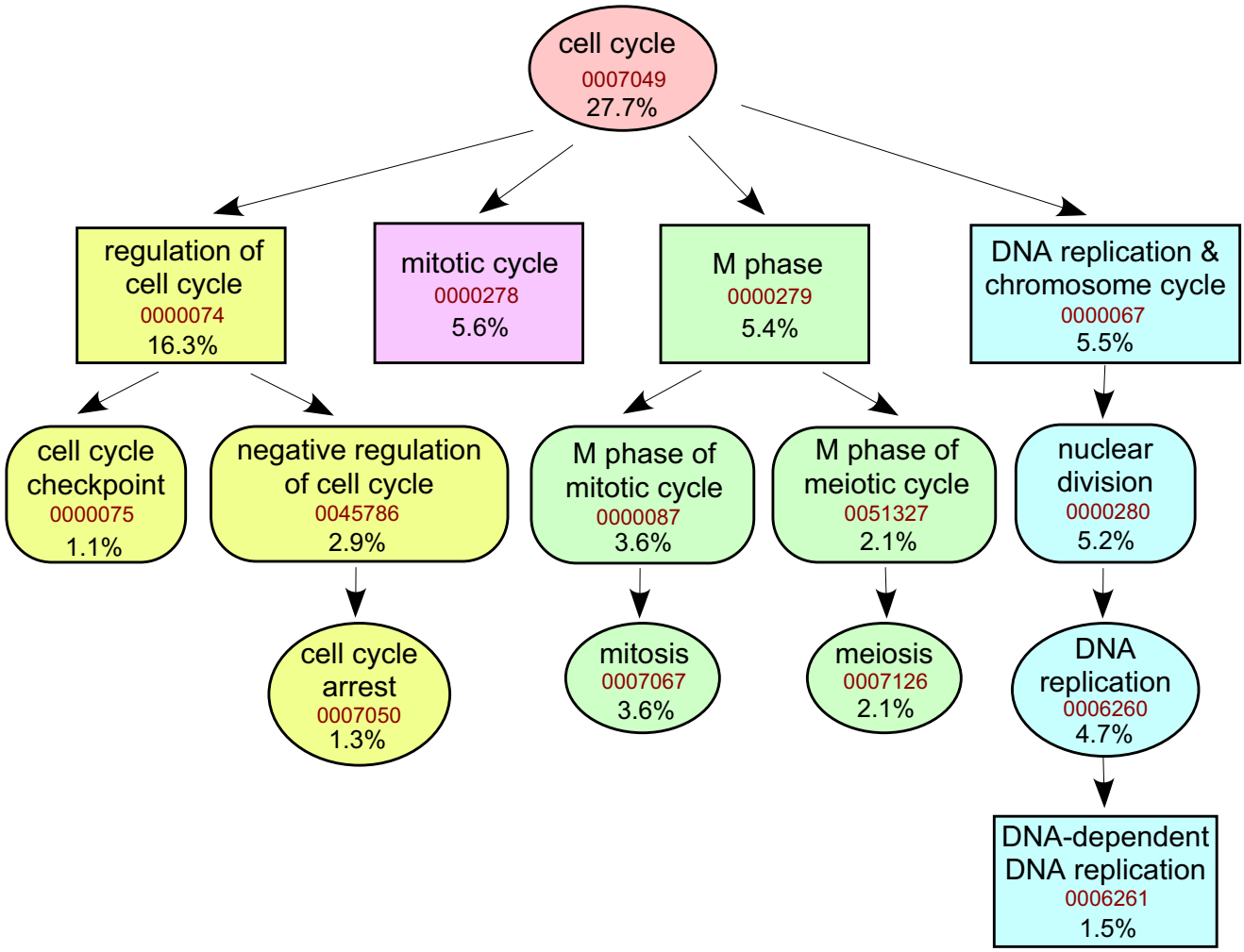

Figure 5
Diagrammatic illustration of various cell cycle-related biological processes in HeLa cells affected by TSPY over-expression, compared to those harboring the vector alone, as revealed by the GOTM program. Each process is represented by the respective GO accession number (red) and percents of genes within the 181 differentially expressed genes used in the GOTM analysis.

SET/NAP domain. In yeast, Nap1 can interact specifically with the B-type cyclin, Clb2, to mediate normal mitotic functions in fission yeast and to suppress polar bud growth in budding yeast [19,20]. The mammalian SET protein can interact with B-type cyclins and has been shown to regulate the G_2/M transition by modulating cyclin B-cyclin-dependent kinase 1 (CDK1) activity [18]. Over-expression of either SET or CDA1 arrested cells at G_2/M phase [12,18], an opposite effect to that of over-expression of TSPY, in HeLa cells, observed here. TSPY protein shares significant homology with these proteins at the SET/NAP domain, but lacks the C-terminal acidic tail that is found in NAP-1, SET and CDA1. Significantly, a recent study suggests that the X-located CDA1/DENTT is a homologue of TSPY and been re-designated as TSPX gene [13]. It shares significant similarities in exon organization, except additional exons at both its 5' and 3' termini. Deletion of the acidic domain in CDA1/TSPX eliminates its inhibitory effects on the G_2/M progression in the cell cycle [12], suggesting the TSPY and TSPX might possess contrasting functions on cell cycle modulation.

The main physiological function of TSPY is currently uncertain. TSPY is expressed in embryonic germ cells and primarily in spermatogonia and to a reduced level the spermatids of adult testis [2,4,8,32]. Spermatogonia are a subset of cells in the testis that are capable of entering both mitotic and meiotic cell divisions [53]. Hence, TSPY

Table 2: List of genes identified in the microarray analysis between HeLa Tet-off cells harboring pTIG-TSPY and those harboring the vector pTIG alone

Gene	GenBank ID	Expression	Remarks
TSPY1	NM_003308	26.5	Testis specific protein, Y-linked, stably transfected gene in the cells
MAP2	NM_002374	3.41	Microtubule-associated protein 2, neurogenesis and tumorigenesis
RGC32	NM_014059	**2.56**	**Response gene to complement 32, deregulation of cell cycle required for tumor cell growth**
PDGFC	NM_016205	**2.32**	**Platelet derived growth factor C, a mitogenic factor**
MAP1B	NM_005909	1.93	Microtubule-associated protein 1B, structural protein, neural development, predictor for breast cancer
EFEMP1	NM_004105	1.79	EGF-containing fibulin-like extracellular matrix protein 1, growth factor
G1P2	NM_005101	1.72	α-interferon inducible protein, IFI-15K, facilitates viral growth
LUM	NM_002345	1.62	Lumican, epithelial cell migration and tissue repair
NANOS1	NM_199461	1.57	Human nanos homolog (of Drosophila), germ stem cell development expressed in spermatogonia and spermatocytes in human testis
WNT5A	NM_003392	1.55	**Wingless-type MMTV integration site family member 5A, Wnt gene family, implicated in oncogenesis and embryogenesis**
CD24	NM_013230	1.55	Small cell lung carcinoma cluster 4 antigen, high expression in SCLC
TMTC1	NM_175861	1.53	Transmembrane and tetratricopeptide repeats containing 1, multi-functional, possibly involved in regulation of cell cycle and/or mitosis.
CCND2	NM_001759	**1.46**	**Cyclin D2 complexes with CDK4 or CDK6, G1/S transition, amplified and over-expresses in numerous types of tumors**
RAP1A	NM_002884	1.44	RAS oncogene family member
EGFR	NM_005228	1.41	Epidermal growth factor receptor (ERBB oncogene), cell proliferation, amplified and expresses at high levels in many tumors
ANKRD15	NM_015158	1.38	Ankyrin repeat domain 15, tumor cell growth in renal cell carcinoma
GSPT1	NM_002094	1.32	G1 to S phase transition 1, an inhibitor of apoptosis
EP300	NM_001429	1.26	E1A binding protein p300, a co-factor for hypoxia inducible factor 1A
CDKN2B	NM_004936	0.71	Cyclin-dependent kinase inhibitor 2B (p15), binds to and prevents CDK4/CDK6 activation and G1/S progression, tumor suppressor
CUL1	NM_003592	**0.68**	**Cullin 1, involves in deneddylation and modulates G1/S transition**
TGFB3	NM_003239	0.67	Transforming growth factor, b3, suppresses tumor formation and blocks cell cycle progression
PTN	NM_002825	0.66	Pleiotrophin, neurite growth promoting factor 1, down-regulated in breast cancer
TIMP1	NM_003254	**0.64**	**Tissue inhibitor of metalloproteinase 1, suppresses tumor growth and metastasis**
DUSP5	NM_004419	0.58	Dual specificity phosphatase 5, inactivates kinases by dephosphorylation, and negatively regulates MAP kinases
SPARC	NM_003118	**0.56**	**Secreted protein, acidc cysteine-rich (osteonectin), loss of expression in lung cancers, regulates cell proliferation**
CLU	NM_001831	0.56	Clusterin, proapoptotic in colon cancer, down-regulated in CaP
IGFBP3	NM_000598	**0.49**	**Insulin-like growth factor binding protein 3, pro-apoptotic, its inactivation or repression is essential for growth of various tumors**

Genes listed in **boldface** were analyzed with semi-quantitative for RT-PCR (Figure 6).

has been postulated to serve a physiological function(s) in stem germ cell proliferation and in directing the spermatogonial cells to enter male meiosis [4,9]. Location of TSPY gene cluster in the critical region for GBY, the only oncogenic locus on the Y chromosome, establishes it to be a significant candidate for this special form of germ cell tumor. Indeed, TSPY has been detected in high levels in gonadoblastoma tissues as well as those of testicular germ cell tumors, more common forms of germ cell tumors [9,24,25]. Additional studies have demonstrated that TSPY is also expressed in prostate cancers and the androgen responsive LNCaP prostate cancer cell line [9,26], hepatoma specimens [30], and melanoma samples and melanoma cell lines [29]. The latter studies further substantiate the possible role of TSPY in human oncogenesis. The present studies were designed to address the question on the effects of ectopic TSPY expression in cell proliferation and tumorigenesis in immunodeficient mice. Using the HeLa, a human (female) cervical carcinoma, and NIH3T3, a non-tumorigenic mouse (lacking a functional Tspy gene), cell lines and the Tet-off transgene regulation system, we demonstrated that over-expression of TSPY potentiates cell proliferation in culture and tumorigenicity in nude mice. Significantly, NIH3T3 cells are non-tumorigenic, the development of small tumors in nude mice inoculated with TSPY expressing NIH3T3 cells suggests that TSPY could potentially play the role of an oncogene. Our cell cycle analyses demonstrated that TSPY was capable of mediating the transition of its host cells through G_2/M phase at a faster pace than those lacking TSPY. These

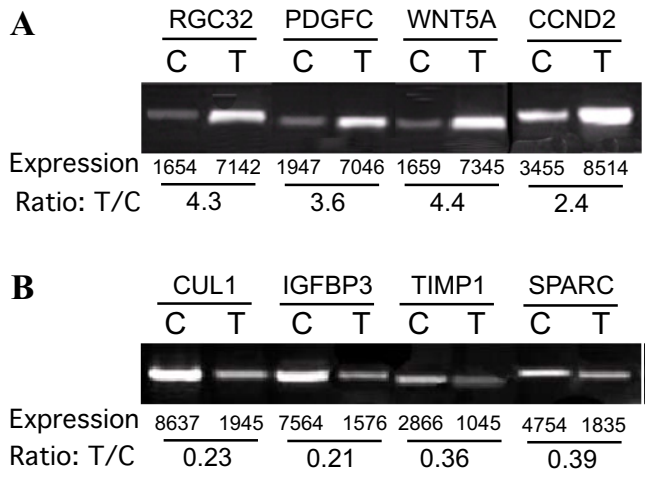

Figure 6
Semi-quantitation RT-PCR analysis of genes up-regulated (A) and down-regulated (B) in HeLa Tet-off cells over-expressing TSPY, using gene-specific primers (Table 1). C = cells stably transfected with vector (pTIG) alone; T = cells stably transfected with TIG-TSPY. The relative expression was calculated by digitization and quantitation software (LabWorks, UVP Inc.). The ratio of T/C represents the relative expression level between the TSPY expressing and control cells for the respective genes (see Table 2).

findings, taken together, support the notion that TSPY is a growth-promoting gene that increases cell proliferation *in vitro* and tumorigenesis *in vivo*, thereby providing a possible explanation of abundant TSPY expression in tumor tissues.

Global transcriptional profiling with microarray analyses further supports the hypothesis that TSPY exerts pro-growth and proliferative functions in the cell cycle progression of its host cells. The up-regulation of such pro-growth genes and oncogenes and down-regulation of growth inhibitors and apoptotic factors in cells over-expressing TSPY, as revealed by the microarray studies, could be confirmed by semi-quantitative RT-PCR analysis. It is also possible that the differential gene expression is a result of indirect effects, perhaps through its interaction(s) with signaling molecules or cell cycle regulators.

Conclusion

Although TSPY expression has been observed in gonadoblastoma, testicular germ cell tumors, prostate cancer, hepatomas, and melanomas, no studies have defined its probable role in human oncogenesis. The present studies have demonstrated that ectopic TSPY expression expedites cell cycle progression through shortening of the G_2/M transition. TSPY also up-regulates pro-growth genes/oncogenes and down-regulates cell cycle inhibitors/apoptotic factors; however, whether this is a direct or indirect effect is unknown. Together, these findings provide a possible mechanism by which TSPY, in collaboration with other oncogenic events, contributes to tumorigenesis in dysfunctional germ cells and/or susceptible somatic cells/tissues.

Abbreviations

TSPY, testis-specific protein Y-encoded; NAP, nucleosome assembly protein; DENTT, differentially expressed nucleolar TGF-β1 target; CDA, cell division autoantigen; CDK, cyclin-dependent kinase; GBY, gonadoblastoma locus on the Y chromosome; CIS, carcinoma in situ; DOX, doxycycline; DMEM, Dulbecco's minimal essential media.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

Y-FCL conceived the hypothesis that TSPY exerts a pro-growth function on cell cycle, supervised and coordinated this study and managed the overall research program on TSPY. SO conceived and performed the studies on growth and colony formation, cell cycle and cell synchronization, and cyclin B1 analysis with respect to cell cycle stages. XXL performed the XTT cell growth and nude mice tumorigenicity assays. TLL and WYC performed the microarray, bioinformatics and semi-quantitative RT-PCR analyses.

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The rat Tspy is preferentially expressed in elongated spermatids and interacts with the core histones ☆

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Abstract

The testis specific protein Y encoded (*TSPY*) gene is a tandemly repeated gene on the mammalian Y chromosome. It encodes several slightly variant proteins that harbor a conserved domain of ~170 amino acids, termed TSPY/SET/NAP1 domain, capable of binding to cyclin B. The human TSPY is preferentially expressed in spermatogonia and to lesser extent in the spermatids. Although rat harbors a single functional *Tspy* gene on its Y chromosome, the human and rat genes differ in their expression patterns, suggesting that they might serve different or variant functions in the testis. Transcripts of *rTspy* were first detected in the testis of 28-day-old rats, at which time the first wave of meiotic division was occurring. The rTspy protein was initially detected in stage-9 elongating spermatids and peaked at stage-13 spermatids in adult testis, but not in spermatogonia, unlike the expression pattern of the human TSPY gene. Using a GST pull-down assay, we demonstrated that rTspy could bind to the core histones H2A, H2B, H3, and H4. Rat Tspy co-localized with the histones in the cytoplasm of selected elongated spermatids. Our results suggest that the rTspy may play critical roles as a histone chaperone during maturation of the elongating spermatids in the rat testis.

Keywords: TSPY; Spermatid; Histone H2A; Histone H2B; TSPY/SET/NAP1 domain; Y chromosome

The testis specific protein Y-encoded (*TSPY*) is an evolutionarily conserved gene on the Y chromosome of mammals, including humans, primates, bovines, and rodents [1–6]. The human *TSPY* (*hTSPY*) and bovine *TSPY* (*bTSPY*) genes are expressed primarily in the spermatogonia of adult testis. TSPY has been postulated to serve a vital function in spermatogonial proliferation and early meiotic division [1,7,8]. Significantly, the human *TSPY* is tandemly repeated 23–64 times and is principally located in the critical region of a cancer predisposition locus, gonadoblastoma on the Y chromosome (GBY), that is associated with gonadoblastoma development in the dysgenetic gonads of XY females and intersex patients [7,9–11]. Indeed, TSPY is strongly expressed in gonadoblastoma tissues and is considered to be the putative gene for GBY [12–14].

In rodents, the mouse harbors an apparently nonfunctional Tspy gene on its Y chromosome with multiple in-frame nonsense mutations in its open reading frame [15,16]. These nonsense mutations seem to be results of recent evolutionary events since numerous related species, such as *Apodemus sylvaticus* [6], do possess a functional *Tspy* gene on their Y chromosome that is capable of encoding sizable proteins with the conserved TSPY/SET/NAP1 domain. The apparent inactivation of the mouse *Tspy* gene suggests that the autosomal *Tspy-like* (*Tspyl*) or other Y-located gene(s) would have assumed its function(s) in germ cell biology. Indeed, there are some examples in the mouse in which autosomal gene has substituted for its homologous human Y-chromosomal gene. One such example is the human heat shock transcription factor on

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Y-chromosome (*HSFY*) gene whose mouse homologue, the *HSFY-like* gene, is located on an autosome, and both genes seem to have an evolutionarily conserved function(s) in spermatogenesis [17]. The rat harbors a functional *Tspy* (*rTspy*) gene that encodes a 334 amino acid protein with the TSPY/SET/NAP1 domain [5,6]. To gain some insights into the function(s) of the rodent Tspy in testis, we had characterized the post-natal expression of the rat *Tspy* gene in the maturing testis using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry analyses. Our results showed that the expression pattern for the rat *Tspy* is distinctly different from that of the human *TSPY* [7,18]. The *rTspy* is specifically expressed in elongating spermatids at late stages of spermatogenesis while the *hTSPY* is expressed preferentially in spermatogonial cells. Cytologically, the rTspy protein was primarily localized in the cytoplasm while the hTSPY shows both cytoplasmic and nuclear locations [18]. Since the expression of the rTspy coincides with the chromatin remodeling stages in which the nuclear histones are being replaced by protamines and exported to the cytoplasm and several members of the SET/NAP proteins are capable of binding to histones in the nucleus involved in gene regulation, we explored the possibility that rTspy might interact with the displaced histones in the cytoplasm. To evaluate this possibility, we had performed additional GST pull-down assays, and demonstrated that the rTspy could interact with the core histones H2A, H2B, H3, and H4, and was co-localized with histone H2B in the cytoplasm of selected elongating spermatids, suggesting that the rTspy may serve a role in spermiogenesis in the rat.

Materials and methods

Animals. Wistar rats and CD1 mice were purchased from the Charles River Laboratories, Inc. (Wilmington, MA). The Institutional Animal Care and Use Committee of the Veterans Affairs Medical Center, San Francisco, approved all experimental procedures in accordance with the NIH Guide for Care and Use of Laboratory Animals.

RT-PCR. Total RNAs were isolated from dissected tissues using the TRIZOL reagent (Invitrogen, Carlsbad, CA) and purified by RQ1-DNase treatment (Promega, Madison, WI) to remove any contaminant DNA. Total RNA (0.36 μg) was reverse-transcribed in a volume of 21 μl using the SuperScript II reverse transcriptase kit (Invitrogen, Carlsbad, CA). One microliter reverse transcribed product was subjected to PCR using a touchdown procedure [18] and specific primer sets for respective genes (Table 1). The PCR products were analyzed by 1.2% agarose gel electrophoresis and visualized with ethidium bromide staining.

Generation of rTspy antiserum. The full-length rat Tspy cDNA was initially isolated from adult testis by RT-PCR with primers rTSPY4F and rTSPY4R, and cloned into the *pGEM-T* Easy Vector System (Promega, Madison, WI). After verification of its sequence, the *rTspy* cDNA was inserted into the *Bam*HI site of *pGEX-4T-3* vector (Amersham Biosciences Corp., Piscataway, NJ). GST–rTspy fusion protein was expressed in bacterial host, BL21DE3, and purified with affinity chromatography using glutathione–Sepharose 4B (Amersham Biosciences Corp, Piscataway, NJ). One milligram of GST–rTspy protein per animal was used to immunize two New Zealand white rabbits at multiple time points to generate polyclonal antibodies using the service of a commercial facility (Sigma-Genosys, Woodlands, TX). The anti-rTspy and pre-immune antisera were further purified by absorbing with GST–Sepharose resins and total mouse testis extracts as described by Conlon and Rossant [19]. The resulting supernatants were used as primary antibodies for immunohistochemistry studies. The respective antisera were confirmed by Western blot with recombinant rTspy protein, as described below.

Western blot analysis. The *rTspy* cDNA was inserted into the *Bam*HI site of mammalian expression vector, *pCS2plus* [20,21], and transfected to COS7 cells using FuGENE6 (Roche, Indianapolis, IN), according to the manufacturer's instructions. The cells were harvested 48 h after the transfection,

Table 1
Sequences of primer sets used in RT-PCR analysis and construction of cDNA

Name	Gene	Direction	Sequence (5′ → 3′)
rTSPY1F	*rTspy* (exon 1)	Forward	ATGGAGAATTCTGAGGAGGAGAGTGTGG
rTSPY1R	*rTspy* (exon 1)	Reverse	GCATTCACAAAGCTGAGCTCCAGTTG
rTSPY4F	*rTspy* (1–334)	Forward	GGATCCATGGAGACTCTAGAGGAGGAGAGTGT
rTSPY4R	*rTspy* (1–334)	Reverse	CGGTGGATCCCGTGAGTGGTCTTCCTTAGGGTAGTAGT
rTSPY5R	*rTspy* (1–96)	Reverse	GCCTCGAGTTAATTCTTGACATCTACCACCTCTGT
rTSPY6R	*rTspy* (1–162)	Reverse	GCCTCGAGTTACACAAAGCTGAGCTCCAGTTG
rTSPY7R	*rTspy* (1–184)	Reverse	GCCTCGAGTTAAAAGTGAGGTCTGCGCATTTT
rTSPY8F	*rTspy* (187–206)	Forward	GATCCCGCAGAAAGACCATAATCCAGGGCATTCCAGGCTTCTGGGCTAAAGCTATGATGAACCATC
rTSPY8R	*rTspy* (187–206)	Reverse	TCGAGATGGTTCATCATAGCTTTAGCCCAGAAGCCTGGAATGCCCTGGATTATGGTCTTTCTGCGG
rTSPY9F	*rTspy* (213–232)	Forward	GATCCATCAGCAACCAAGATGAAGACTTACTGAGCTACATGTTGAGCTTGGAGGTGGAGGAGTACC
rTSPY9R	*rTspy* (213–232)	Reverse	TCGAGGTACTCCTCCACCTCCAAGCTCAACATGTAGCTCAGTAAGTCTTCATCTTGGTTGCTGATG
Ssty1F	*Ssty1*	Forward	AAAGGCAAGCCAGCTCCTGAACTCCA
Ssty1R	*Ssty1*	Reverse	TTCCATTGGGTGACAGGCTCATTACC
Eif2s3yF	*Eif2s3y*	Forward	AGCCGCATCTTTCTCGTCAGGATCTT
Eif2s3yR	*Eif2s3y*	Reverse	TTCAATGGCAGCCAGGTGTTCAGAAG
TH2BF	*Testis H2B*	Forward	GCGAATTCTATGCCGGAGGTGTCGGCAAAGGGG
TH2BR	*Testis H2B*	Reverse	GCGGATCCTCACTTGGAGCTGGTGTACTTGGT
β-actin5	β-*actin*	Forward	TCACCCACACTGTGCCCATCTACGA
β-actin2R	β-*actin*	Reverse	CCACGTCACACTTCATGATGGA

washed with PBS, and lysed with 100 μl TNBT buffer containing 50 mM Tris–HCl (pH 7.0), 150 mM NaCl, 1% NP-40, and 0.25% deoxycholic acid. Five microliters of cell lysates or 50 μg of rat testis extracts was subjected to SDS–PAGE on 12% (w/v) polyacrylamide gel and processed for Western blot using a semi-dry transfer method [22] and the purified rTspy antiserum. The binding of the primary antibody was detected with a peroxidase-conjugated anti-rabbit IgG antibody and visualized by the ECL-plus chemiluminescence system (Amersham Biosciences Corp., Piscataway, NJ) or diaminobenzine (DAB) (Vector Laboratories, Burlingame, CA).

Histology and immunohistochemistry. Rat tissues were fixed for 12 h in Bouin's fluid (Sigma–Aldrich, St. Louis, MO) or paraformaldehyde–PB solution, 4% (w/v) paraformaldehyde, 100 mM phosphate buffer (pH 7.4) at 4 °C, dehydrated in ethanol series, and embedded in paraffin wax. Five micrometer sections were dewaxed and rehydrated through alcohol series and finally in distilled water. Antigen retrieval was performed in 100 mM Tris–HCl (pH 10) at 95 °C for 30 min. The sections were incubated in 3% hydrogen peroxide, washed in PBS, and nonspecific protein binding was blocked using 3% BSA–PBS solution. Sections were incubated overnight in a humid chamber at 4 °C with anti-rTspy (1:400) or anti-histone H2B (1:200, Upstate, Charlottesville, VA) antibody. The immunoreactive sites were detected with the SuperPicTure Polymer Detection kit (ZYMED/Invitrogen, Carlsbad, CA) and sections were counterstained with hematoxylin (Fisher Scientific, Hampton, NH). For identification of rTspy-positive germ cells, the Tyramide Signal Amplification Kit (with biotin-XX; Molecular Probes/Invitrogen, Carlsbad, CA) was used to amplify the immunoreacting signals following the incubation with the SuperPicTure Polymer Detection kit.

Immunofluorescence was performed with paraformaldehyde-fixed sections and processed similarly, as described above. The signals were visualized by Alexa488 conjugated anti-rabbit IgG (Molecular Probes/Invitrogen). For double immunofluorescence, the anti-rTspy and anti-H2B antibodies were directly labeled in red and green, respectively, by Zenon Rabbit IgG Labeling Kit (Molecular Probes/Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. DNA was stained with DAPI (Roche, Indianapolis, IN).

GST pull-down assay. Various rat Tspy cDNAs harboring different lengths of its coding sequences were initially generated by PCR with the full-length rTspy cDNA and primers (Table 1), and cloned into the *pGEM-T* Easy Vector System (Promega, Madison, WI). After verification of their sequences, the cDNAs were individually inserted between *Bam*HI and *Xho*I sites of the expression vector, *pGEX-4T-3*. The *hTSPY* cDNA [3] was inserted in the plasmid *pGEX-4T-3* and processed similarly for fusion protein production in bacterial host. Approximately 250 μg GST, GST–rTspy or GST–hTSPY proteins was bound separately to glutathione–Sepharose 4B (Amersham Biosciences Corp., Piscataway, NJ) and reacted independently with 5 μg of histone H2A or H2B (Upstate Biochemicals, Waltham, MA) in 100 μl of buffer A (PBS, 20% glycerol, 0.1% NP40, 5 mM DTT, and proteinase inhibitors with EDTA [Roche]) overnight at 4 °C. The GST pull-down assays with histones H3 and H4 (Upstate, Waltham, MA) were performed similarly with modified buffer A, containing additional 1% NP40 and 250 mM NaCl to reduce nonspecific binding of GST. Protein complexes bound to the Sepharose beads were precipitated by centrifugation and washed with 500 μl of buffer A for 10 min. Proteins were eluted with 40 μl of SDS sample buffer, analyzed by SDS–PAGE on 10–20% (w/v) polyacrylamide gradient gels (Bio-Rad Laboratories, Hercules, CA), and visualized by Coomassie blue staining or Western blot with appropriate antibodies (Upstate, Waltham, MA). The relative amounts of bound and unbound histones were estimated by the NIH Imager Program (http://rsb.info.nih.gov/nih-image/index.html).

Immunocytochemical analysis. The C-terminal truncated rat Tspy cDNAs, rTspy (1–96), and rTspy(1–162), were individually inserted between *Bam*HI and *Xho*I sites of the mammalian expression vector, *pCS2plus*. The testis type histone H2B (TH2B) cDNA was initially isolated from adult testis by RT-PCR with primers TH2BF and TH2BR (Table 1), and cloned into the *pGEM-T* Easy Vector System. After sequence verification, the cDNA was inserted between *Eco*RI and *Bam*HI sites of the green fluorescence protein expression vector *pEGFP-C1* (Clonthech Laboratories, Mountain View, CA) to express EGFP–TH2B fusion protein. The resultant *pCS2-rTspy* variants and/or *pEGFP-TH2B* plasmid were transiently transfected to COS7 cell using FuGENE6. Forty-eight hours after the transfection, the cells were fixed by 10% formalin–PBS solution for 5 min, and processed for immunofluorescence with the anti-rTspy antiserum and anti-EGFP mouse IgG (Clontech Laboratories, Mountain View, CA). The immunoreactive signals were detected by Alexa594-conjugated anti-rabbit IgG and Alexa488 conjugated anti-mouse IgG (Molecular Probes/Invitrogen, Carlsbad, CA) and analyzed by fluorescence microscopy.

Results

Developmental expression of rTspy in post-natal testes

The tissue-specific and developmental expression of the *rTspy* was analyzed by RT-PCR. The open reading frame

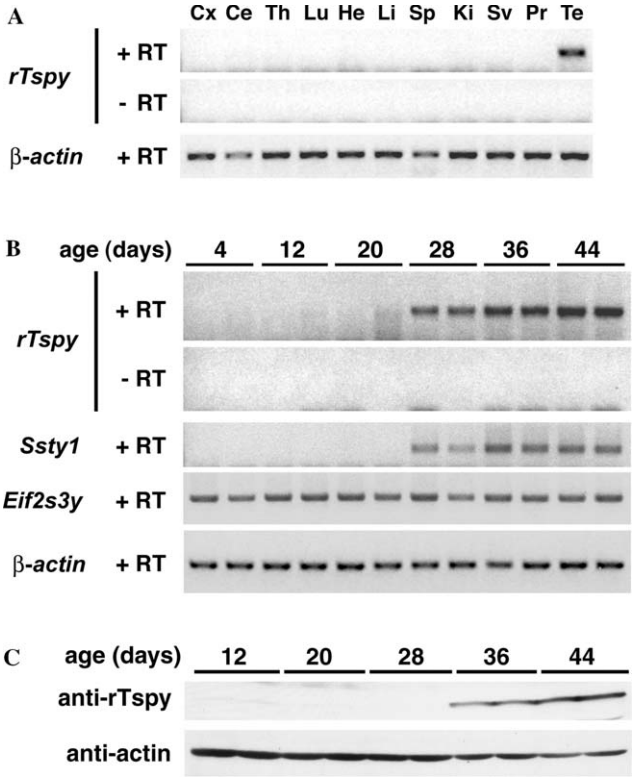


Fig. 1. The tissue specific expression in rat Tspy in developmental and adult testes. (A) Tissue specificity was analyzed by RT-PCR. Tspy was detected only in adult rat testes. (B) Tspy mRNA expression in rat post-natal testes. Total RNAs from testes of 2 rats at 4, 12, 20, 28, 36, and 44 days of age were individually analyzed by RT-PCR with a primer set derived from exon 1 of the rat *Tspy* gene. Significant signals were detected in the testes of 28 days and older rats. The expression of rat *Ssty1* and *Eif2s3y* was analyzed in parallel with *rTspy*. In the case of the mouse, *Ssty1* is expressed in elongated spermatids while *Eif2s3y* is expressed in spermatogonia and prophase spermatocytes. The similar patterns between the *rTspy* and *Ssty1* suggest that they are expressed in similar manner in the post-natal rat testes. (C) Tspy protein expression in post-natal rat testes. Total proteins from testes of 2 rats at 12, 20, 28, 36, and 44 days of age were analyzed by Western blot by using anti-rTspy and anti-actin antibodies, respectively. Significant signals were detected in the testes of 36 days and older rats. *Abbreviations:* Cx, cerebral cortex; Ce, cerebellum; Th, thymus; Lu, lung; He, heart; Li, liver; Sp, spleen; Ki, kidney; Sv, seminal vesicle; Pr, prostate; Te, testis; +RT, with reverse transcription; −RT, without reverse transcription. β-*Actin* was used as a positive control for all samples.

of the *rTspy* transcript encodes a protein with 334 amino acids spanning from exon 1 to exon 5 (Genbank Accession No. AF074880) [5]. In adult animals, *rTspy* transcripts were detected abundantly in testis, but not in other tissues examined (Fig. 1A), consistent with the results of Dechend et al. [5]. To determine the overall time course of *rTspy* expression in developing testis, similar studies were conducted with total RNAs derived from two male rats each at ages 4, 12, 20, 28, 36, and 44 days. Our results showed that *rTspy* expression was initially detected in testes of rats at 28 days of age, when the first wave of meiotic division occurred. The level of *rTspy* expression increased with age of the animals (Fig. 1B, *rTspy*). As references, expression of the Y-located *Ssty1* and *Eif2s3y* genes was analyzed similarly. In the mouse, *Ssty1* is expressed specifically in spermatids [23,24] while *Eif2s3y* is primarily expressed in spermatogonial cells and germ cells through the meiotic prophase [25]. Similar to *rTspy*, transcript or the rat *Ssty1* was detected only at or after 28 days of age while *Eif2s3y* transcript was more ubiquitous among testes of different ages (Fig. 1B, *Ssty1* and *Eif2s3y*), reflecting their spermatid and spermatogonia/early germ cell expression patterns, respectively. The authenticity of the amplified products was confirmed to be those of *Ssty1* and *Eif2s3y*, respectively, by DNA sequencing analysis of the RT-PCR products (data not shown). Our results suggested that *rTspy* mRNA was expressed specifically in haploid germ cells.

The developmental expression of rTspy protein was analyzed by Western blot with testicular protein extracts from 12-, 20-, 28-, 36-, and 44-day-old rats. The result showed that rTspy protein was initially detected in the testis of 36-day-old rats (Fig. 1C, anti-rTspy), while the *rTspy* mRNA was detected in those at 28 days and later (Fig. 1B, *rTspy*). The most differentiated type of germ cells was spermatocytes, round spermatids, and elongating spermatids of 20-, 28-, and 36-day-old rats, respectively. Our results, taken together, suggested that *rTspy* is initially transcribed in round spermatids and translated in elongated spermatids.

The rTspy is specifically located in the elongated spermatids in adult testis

To confirm the results obtained from Western blot of developmental testes, we had performed an immunochemical staining study on adult rat testis using a polyclonal antiserum against the rTspy protein. The specificity of this antiserum was initially demonstrated by Western blot of total protein lysates derived from COS7 cells transfected with rTspy expression vector (Fig. 2I, lane 2, arrowhead). The respective rTspy band could be abolished when the antiserum was pre-absorbed with excess GST–rTspy recombinant protein (Fig. 2I, lane 4). Although the conserved TSPY/SET/NAP1 domain is also present in the products of other members of the TSPY/TSPY-like/SET/NAP-1 (TTSN) superfamily (e.g. Tspy-like protein 1 [26] and DENTT/Tspy-like 2 protein [27]), no cross reactivity with hTSPY on Western blot was observed (data not shown), suggesting that our anti-rTspy antiserum was specific for the rTspy protein.

Immunohistochemical analysis was performed on sections of rat testes fixed in Bouin's fluid. To minimize any non-specific reactivity, we treated the antiserum with total mouse testis protein extract before using it in the immunostaining procedure. Although the mouse *Tspy* gene was transcribed, its transcripts were incapable of coding for the full-length or any protein of significant size. Hence, we did not anticipate any depletion of the rTspy antibodies in antiserum with this pre-absorption procedure. Using this processed rTspy antiserum, we observed specific immunostaining in the elongating spermatids at spermatogenic stages XIII–XIV (Fig. 2A, arrowheads in stage XIII–XIV, and Fig. 2J [28]). Again, the immunostaining was significantly decreased by pre-absorption with GST–rTspy (Fig. 2B, blue arrowheads). The Sertoli cells and germ cells from spermatogonia to stage 7 round spermatids did not react with this antiserum (Fig. 2D–G; Sg, Zc, Pc, Di, Rd, and Se; Fig. 2J). The immunostaining signals were detected faintly in stage 8 spermatids that were in the process of nuclear elongation (Fig. 2E, blue arrowheads; and J). They reached maximal levels in stage 12 elongating spermatids (Fig. 2D, blue arrowheads), but rapidly decreased and became minimally detectable at stage 16–18 spermatids (Fig. 2G, blue arrowheads; Fig. 2J). The interstitial cells including Leydig cells showed some non-specific staining (Fig. 2A, black arrows) that did not diminish even after a pre-absorption with GST–rTspy (Fig. 2B, black arrows). Our results, taken together, suggested that the rTspy protein initially expressed in spermatids at approximately stage 8, peaked at in stage 12–14 elongating spermatids, and decreased during spermatogenic stage II to V elongated spermatids (Fig. 2J, blue lines; modified from Russell et al. [28]).

rTspy and hTSPY bind directly to core histones H2A, H2B, H3, and H4

The human TSPY and rat Tspy proteins harbor a conserved ~170 amino acid domain, known as the TSPY/SET/NAP1 domain, shared by numerous members of this protein superfamily, represented by TSPY, SET oncoprotein, and nucleosome assembly protein 1 (NAP1) (NCBI Conserved Domain Search #pfam00956 [29]) [26,30]. This conserved domain is located at residues #150–328 of the rTspy and residues #110–288 of the hTSPY (Fig. 3A). Numerous studies suggested that the proteins containing the TSPY/SET/NAP1 domain are capable of binding to either the mitotic cyclin B [31,32] or core histones [33–35], and are involved in cell cycle regulation and the nucleosome assembly and/or chromatin remodeling, respectively. In particular, SET/TAF-Iβ can serve as a subunit of the inhibitor of acetyltransferases (INHAT). It binds to the core histones H2B, H3, and H4 in the nucleus and either inhibits enzymatic activities of acetyltransferases

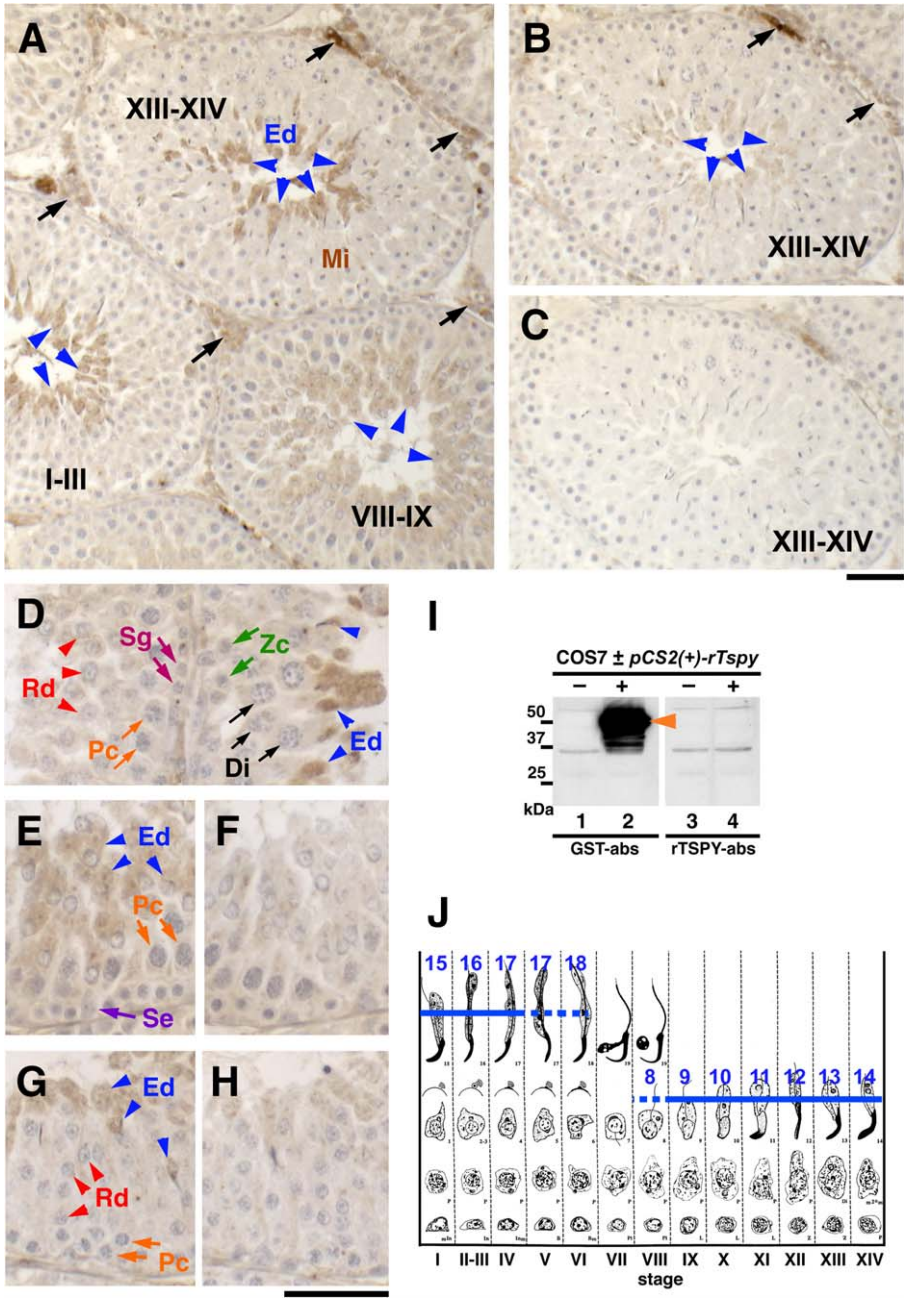


Fig. 2. Detection of spermatid-specific expression of rat Tspy using immunohistochemistry. (A) Only elongating spermatids at various spermatogenic stages were stained positive for the anti-rTspy antiserum (arrowheads). Roman numbers indicate spermatogenic stages as described in (J). (B) Image of an adjacent section immunostained similarly by a control antiserum preabsorbed with GST–rTspy. The staining of elongating spermatids was significantly reduced (arrowheads). (C) Image of an adjacent section immunostained by a preimmune serum as a negative control. (D) Elongating spermatids at stage XIII are positively stained (blue arrowheads), but spermatids of stage II and III are negative (red arrowheads). No other cells, including somatic cells, were positive for the antiserum (Sg, Zc, Pc, and Di). (E–H) Sections of seminiferous tubules at spermatogenic stage VIII (E and F) and II–IV (G and H) immunostained by the anti-rTspy antiserum (E and G), and control antiserum preabsorbed with GST–rTspy (F and H). Only elongating spermatids are faintly positive (blue arrowheads). *Abbreviations:* Di, diplotene spermatocytes; Ed, elongating spermatids; Mi, meiotic dividing cells; Pc, pachytene spermatocytes; Rd, round spermatids; Se, Sertoli cells; Sg, spermatogonia; Zc, zygotene spermatocytes. Scale bars = 50 μm in A–C and D–H, respectively. (I) Western blot of COS7 cells transfected with a rat Tspy expression vector using the anti-rTspy antiserum pre-absorbed with GST alone (lanes 1 and 2; GST-abs) and with GST–rTspy fusion protein (lanes 3 and 4; rTspy-abs). Positive band for rat Tspy protein was detected in COS7 cells transfected with rat Tspy expression vector (lane 2), but not in non-transfected cells (lanes 1 and 3) or those reacted with the same antiserum subtracted with GST–rTspy protein (lanes 3 and 4). Anti-rTspy serum detected recombinant rTspy (lane 2). (J) Spermatogenic stages of the rat testis. Blue lines indicate the immunopositive cells in steps 8–18 of spermatid development. Roman numbers indicate stages of spermatogenesis. Dotted lines represent sections showing faint staining of spermatids in these stages. Modified from review by Russell et al. [28].

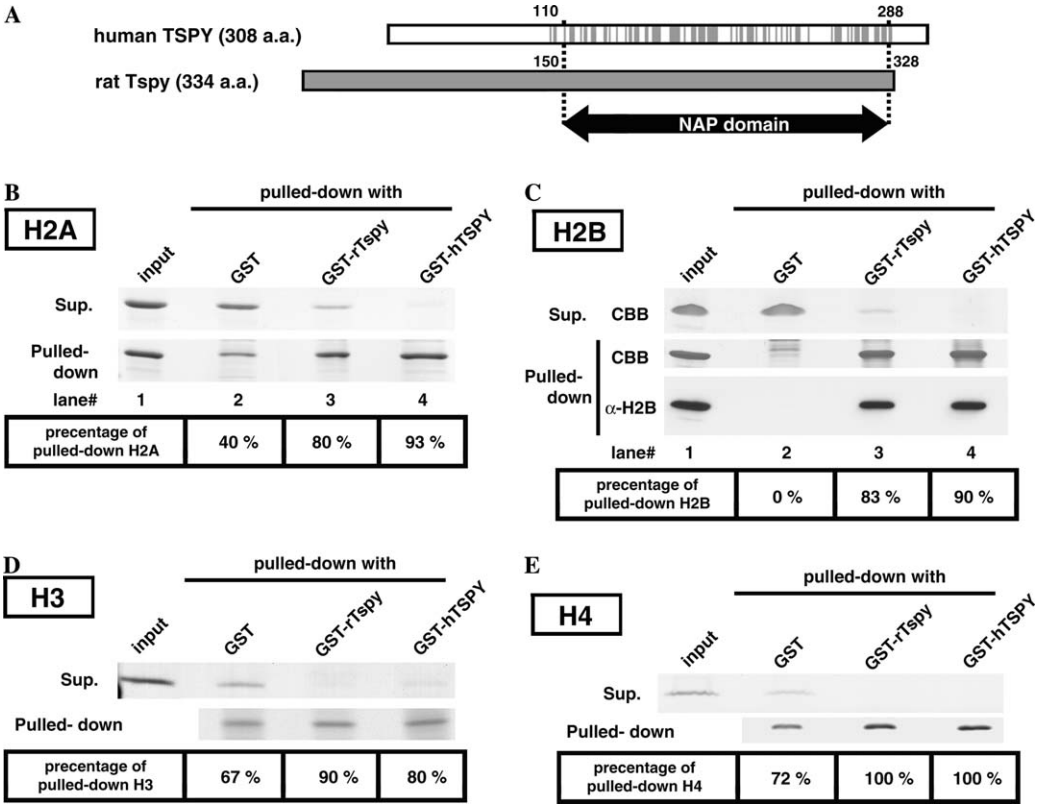


Fig. 3. Interactions of rTspy and hTSPY with histones H2A, H2B, H3, and H4. (A) Alignment of hTSPY and rTspy proteins showing the homologous region encompassing the TSPY/SET/NAP1 domain. Gray lines on the hTSPY indicate positions with identical amino acid as the rTspy. The ~170 amino acid TSPY/SET/NAP1 domain is located between residue #110–288 of the hTSPY and residue #150–328 of the rTspy. The TSPY/SET/NAP1 domain is indicated by arrow (NAP domain). (B) GST pull-down assay of histone H2A using GST or GST-fusion proteins as indicated. Pulled-down proteins (pulled-down) and proteins of supernatant (supernatant) were subjected to SDS–PAGE and visualized by Coomassie blue staining. Histone H2A was preferentially retained by Sepharose 4B resins conjugated with either GST–rTspy (lane 3) or GST–hTSPY (lane 4) but not by those conjugated with GST alone (lane 2). The numbers on bottom column indicate the approximate percentage of pulled-down H2A to total amount of H2A. Input represents equivalent material used for each GST pull-down assay. (C) GST pull-down assays of histone H2B using GST-fusion proteins. The experiment was performed similarly as described in (B). Both GST–rTspy and GST–hTSPY fusion proteins (lanes 3 and 4, respectively) showed significant affinity to histone H2B than GST alone (lane 2). The input represents the material used for GST pull-down assay. The authenticity of the pulled-down material as H2B was confirmed by Western blot with anti-H2B antibody (bottom row, α-H2B). (D,E) GST pull-down assay of histones H3 (D) and H4 (E) performed similarly as described in (B). *Abbreviations:* CBB, Coomassie blue-stained band; α-H2B, Western blot with anti-H2B antibody.

or facilitates transcription of target genes [36–38]. Since transcriptional activities of the elongating spermatids are drastically reduced as their chromatin is undergoing compaction, it is uncertain if the rTspy is involved in any transcription modulation. However, chromatin compaction is usually accompanied by replacement of histones by protamines in the nucleus and exportation of the replaced histones to the cytoplasm of maturing spermatids. Although the cytoplasmic location of the rTspy protein would have excluded it as an active participant of any transcriptional process, it raises the possibility that rTspy might interact with the displaced histones in the cytoplasm.

To evaluate this possibility, we performed a GST pull-down study between the rTspy/hTSPY and various purified core histones. This initial study demonstrated that core histones H2A, H2B, H3, and H4 were preferentially bound to GST–rTspy or -hTSPY fusion proteins, as compared to binding with GST alone (Fig. 3B–E). Western blot analysis using anti-histone H2B antibody confirmed our observations that this histone was preferentially pulled down by both GST–rTspy and GST–hTSPY baits (Fig. 3C, α-H2B). These results suggest that both rTspy and hTSPY are capable of binding directly with the core histones H2A, H2B, H3, and H4.

It was reported that SET/TAF-Iβ interacts with histones through its C-terminal acidic tail [39,40]. Although neither rTspy nor hTSPY has such a long acidic tail, as in SET/TAF-Iβ, rTspy as a whole is highly acidic (with a predicted p*I* of 4.39). Rat Tspy contains abundant aspartic acid and glutamic acid residues, within its N-terminal region (Fig. 4A). Hence, we tested the histone binding ability for the N-terminal region and the acidic region of NAP-domain by GST-pull-down assay using histone H2B as a probe. The N-terminal region of rTspy bound strongly to histone H2B at the similar level as that for the full-length rTspy (Fig. 4B, lanes 3 and 7). The acidic region in the TSPY/SET/NAP1-domain (#213–232) also bound histone H2B, but at a weaker level than that of the full-length

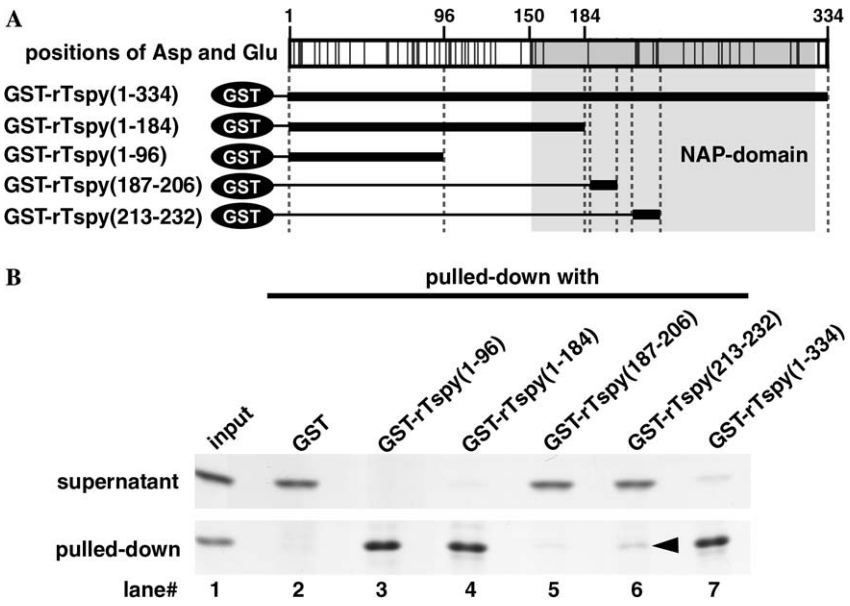


Fig. 4. Analysis of the histone H2B binding region in rTspy. (A) Simplified structure of GST-fusion proteins used in GST pull-down assay. Top panel indicates the positions of aspartic acid and glutamic acid in rTspy. The positions of acidic amino acid are indicated by black lines. NAP-domain is shadowed by gray. In lower panel, bold lines indicate the GST-fused region analyzed in (B). (B) GST pull-down assay of histone H2B using GST or GST-fusion proteins as indicated. Pulled-down proteins (pulled-down) and proteins of supernatant (supernatant) were subjected to SDS-PAGE, and visualized by Coomassie blue staining. The input represents the material used for GST pull-down assay.

rTspy (Fig. 4B, lane 6). Non-acidic region of NAP-domain (#187–206), however, did not bind histone H2B with similar assay (Fig. 4B, lane 5). Based on these results, we postulate that rTspy preferentially interacts with the core histones at the N-terminal portion of its protein.

Rat Tspy co-localizes with histone H2B in the cytoplasm of elongating spermatids

We further explored the possibility that both rTspy and displaced histones were co-localized in the cytoplasm of the spermatids using immunofluorescence and individually labeled primary antibodies. Our results showed that positive signals for H2B were detected in the nuclei of elongated spermatids at spermatogenic stage XI–XII (Fig. 5A and C, arrowheads), but not detectable in the nuclei of elongated spermatids at spermatogenic stage XIII–XIV (Fig. 5B and D, arrowheads). Under high magnification, histone H2B was exclusively localized in the cytoplasm and co-localized with rTspy in the elongated spermatids at spermatogenic stage XII–XIII (Fig. 5E–G, arrowheads). Immunohistochemical staining of adjacent sections of the rat testis independently with anti-rTspy and anti-H2B antibodies confirmed that H2B and rTspy were indeed co-localized in the cytoplasm of the elongating spermatids at stage XIII–XIV (Fig. 5H and I, arrowheads). The cytoplasmic localization of histone H2B was initially detected in stage 11 elongating spermatids, and reached a maximal level at stage 13–14 elongating spermatids in which rTspy protein was also at its maximal level (Fig. 5K). These observations suggested that rTspy was expressed at a period of spermatogenesis in which the core histones were being replaced by protamines and exported to the cytoplasm of maturing spermatids. The interaction between the rTspy and core histones (Fig. 3) raised the possibility that it might serve a role in the processing of the discarded core histones during spermiogenesis. Although the hTSPY is primarily located in the spermatogonia [7], our recent study showed that it could also be expressed in spermatids of adult testis [18]. The fact that it also interacts with core histones suggests that it might serve similar function(s) as that of the rat Tspy protein in spermiogenesis.

The TSPY/SET/NAP1 domain of rTspy regulated/restricted the intracellular localization of rTspy in COS7 cells

To further explore the interaction between rTspy and histones, rTspy of various lengths and/or EGFP-testis type H2B fusion protein (EGFP-TH2B) were either singly or doubly transfected to COS7 cells, and analyzed for the subcellular locations of their respective proteins by immunofluorescence. The full-length rTspy was preferentially localized in cytoplasm consistent with the location of the endogenous rTspy protein in elongating spermatids (Fig. 6C). Interestingly, the rTspy(1–96), harboring the histone H2B binding site but not the TSPY/SET/NAP1 domain (Fig. 4), was distributed evenly in both nucleus and cytoplasm of transfected cells (Fig. 6A). In cells co-transfected with rTspy(1-96) and EGFP–TH2B, we found that rTspy(1–96) was well co-localized with the EGFP–TH2B on selected areas within the nuclei (Figs. 6D–F and Supplemental Fig. S2 for control). Although some cells

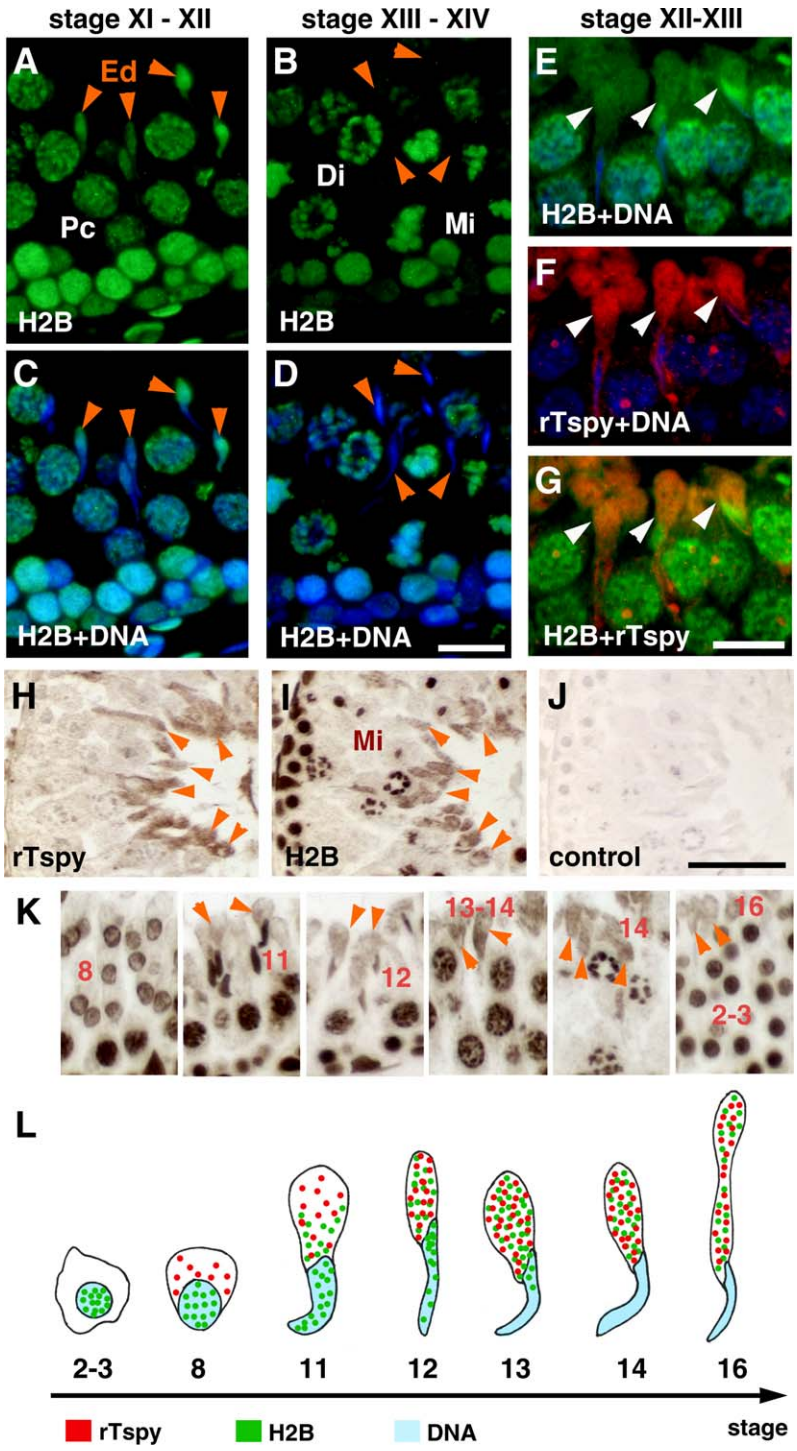
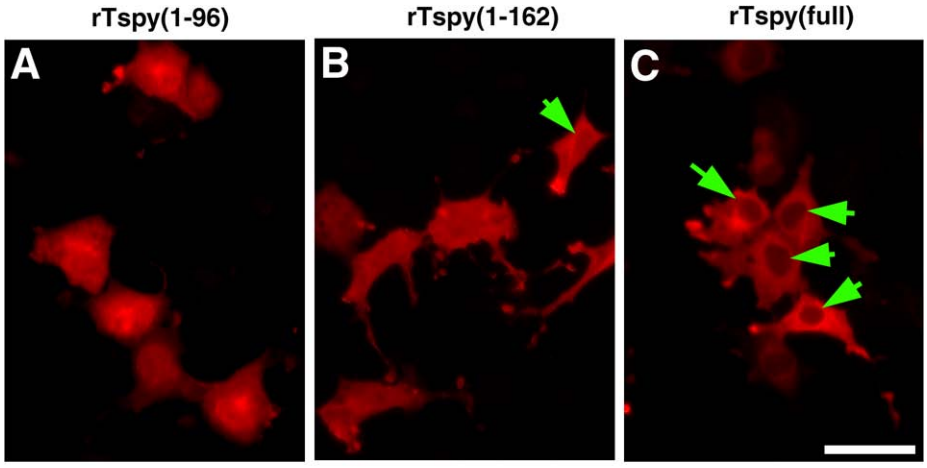


Fig. 5. Co-localization of histone H2B and rTspy on elongated spermatids of adult rat testis. (A–D) Immunofluorescence of seminiferous tubules at stage XI–XII (A,C) and stage XIII–XIV (B,D) using anti-H2B antibody. DNA was stained with DAPI. (C) and (D) represent the merged images of H2B (green) and DNA (blue). Histone H2B was excluded from nuclei of elongated spermatids at stage XII–XIV. (E–G) Immunofluorescence of seminiferous tubules at stage XII–XIII doubly immunostained with individually labeled H2B and rTspy antibodies. The merged images of H2B (green) and DNA (blue) (E), rTspy (red) and DNA (blue) (F), and H2B (green) and rTspy (red) (G), respectively. The cytoplasm of elongating spermatids was positive for both H2B and rTspy, and is represented as yellow/orange fluorescent signals in the merged image (arrowheads in G). *Abbreviations:* Di, diplotene spermatocytes; Mi, meiotic dividing cells; Pc, pachytene spermatocytes. (H) Rat testis immunostained by anti-rTspy antiserum. Cytoplasm of elongated spermatids was positively stained (arrowheads). (I) Adjacent section of H that was immunostained by anti-H2B antibody. The cytoplasm of elongating spermatids (arrowheads), and the nuclei of other cells were stained. Arrowheads indicate the identical cells between (H) and (I). (J) Image of the rat testis processed with the secondary antibody alone for negative control. Scale bars = 50 μm in (A–D); 25 μm in (E–G); 50 μm in (H–J). (K) Immunostaining of histone H2B in various stages of spermatids. Arabic numbers indicate the stages of respective differentiating spermatids. Orange arrowheads point to the immunoreactive cytoplasm of various spermatids. (L) A diagram illustrating the expression and localization of rTspy (red dots) and histone H2B (green dots) in adult rat testis. Arabic numbers indicate the stages of spermatids. The locations of DNA/nuclear are represented by blue color.



H

	rTspy(1-96)	rTspy(1-162)	rTspy(full)
Whole cell distribution	89 %	69 %	10 %
Cytoplasmic distribution	11 %	31 %	90 %

Fig. 6. Intracellular localization of rTspy proteins of various lengths in COS7 cells. (A–C) Immunofluorescent images of COS7 cells singly transfected with *pCS-rTspy(1–96)* (A), *pCS-rTspy(1–162)* (B) or *pCS-rTspy(full-length)* (C) using anti-rTspy antiserum (red). Arrows indicate less fluorescence at the nuclei than the cytoplasm of transfected cells. Scale bar = 50μm. (D–G) Immunofluorescent images showing intranuclear localization of rTspy(1-96) (D) and EGFP–TH2B fusion protein (E) in the COS7 cell co-transfected with *pCS-rTspy(1–96)* and *pEGFP–TH2B*. The signal of EGFP–TH2B was enhanced by anti-EGFP antibody. The dots of rTpsy(1–96) (red) co-localized well with those of EGFP-TH2B (green), as represented in yellowish color in the merged image (F). DNA was visualized by DAPI staining (G). (H) Percentage of COS7 cells that displayed whole cell distribution (equal intensity between nuclei and cytoplasm) and preferentially cytoplasmic distribution of respective rTspy variants. For each construct, ~300 cells were randomly counted.

transfected with a slightly longer construct expressing the rTspy(1–162) including 12 amino acid of TSPY/SET/NAP1 domain, might show some cytoplasmic locations of the gene products, most of the rTspy(1–162) still remained in nuclei, as compared with the full-length rTspy (Fig. 6B and H). Hence, the TSPY/SET/NAP1 domain of rTspy could be important on restricting/regulating the cytoplasmic localization of the full-length rTspy. These observations support the hypothesis that rTspy interacts with the displaced histones in the cytoplasm of elongating spermatids.

Discussion

The *Tspy* gene is evolutionarily conserved and located on the Y chromosome of all mammalian species examined [1,2,4,5]. However, besides the TSPY/SET/NAP1 domain, it diverges considerably along the evolutionary tree. The human *TSPY* gene is tandemly repeated in 20.4-kb repeat units that encompass ~0.7 MB DNA on the short arm of the Y chromosome and constitute the largest block of functionally repeated genes in the human genome [41]. Such repetitiveness of the gene has been conserved among some mammalian species, such as bovine and equine genomes [1]. However, in the rodents, including species of the *Apodemus*, *Mus*, and *Rattus*, the functional Tspy gene is a single-copy gene [42]. In particular, the presence of nonsense mutations along its putative coding sequence in the laboratory mice, i.e. *Mus musculus* and *Mus musculus domesticus*, suggests that this gene might be undergoing a decaying process(es) on the Y chromosome [6,15]. The rodent *Tspy* gene, in addition to its sequence divergence with the human *TSPY* gene, exhibits an expression pattern, as reported here, different from those of the human *TSPY* or bovine *Tspy* genes. The preferential expression of the human *TSPY* in embryonic germ cells and spermatogonia

in adult testis suggests that it might exert a critical effect(s) on germ cell proliferation and meiotic division [1,7]. On the other hand, the preferential spermatid expression of the rat *Tspy* in adult testis suggests that the rodent *Tspy* gene might play an important role in the spermiogenesis process. Our recent demonstration of detectable expression of the human TSPY in spermatids of adult testis [18] indicates that it might also play similar role as the rodent Tspy in spermatozoa maturation. Hence, it is reasonable to assume that the human TSPY may play a variety of roles in germ cell proliferation, meiotic division, and spermiogenesis. The rodent, i.e. the rat, Tspy could have lost its role in germ-cell proliferation and meiotic division, but retains that in spermiogenesis.

Currently the exact mechanism(s) of such differential expression between the human *TSPY* and the rat *Tspy* gene is uncertain. Preliminary sequence analysis of the putative rat *Tspy* promoter region revealed that it is significantly different from that of the human *TSPY* gene. The rat *Tspy* promoter contains a CRE element located at 50-bp upstream of the transcription start site (Supplemental Fig. S1). Such element is absent in the human *TSPY* gene promoter. The transcription factor, CREM, binds to the CRE element and mediates post-meiotic transcription of many spermatid-specific genes [43], including *protamine 2*, *TP1*, and the Y-located *Ssty1* [43–46]. The promoters of many of these spermatid-specific genes, including the rat *Tspy*, do not harbor any TATA box, but contains the CRE element (Supplemental Fig. S1). Mice homozygous for a CREM null allele showed an arrest of their spermatogenesis at the haploid germ cell stage [47]. The promoter for the human *TSPY* gene is also TATA-less, but is rich in CpG sequences. The human *TSPY* promoter is subjected to methylation that regulates its expression in certain cancer cells [48]. Despite such differences between the promoters of the rat *Tspy* and human *TSPY*, the exact mechanism(s) of their differential expression patterns cannot be unambiguously defined since various transgenic mouse studies showed variation of transgene expression patterns by the human *TSPY* promoter [18,49,50]. Transgenic mice harboring a human 2.4-kb *TSPY* promoter-directed reporter gene showed a preferential transgene-expression in spermatids of the testis, similar to the rTspy expression pattern in the present study [18]. Another study suggested that the SV40 large T antigen oncogene directed by a 1.3-kb human TSPY promoter was capable of inducing tumor formation in the pituitary gland of the host [49]. Other transgenic mice harboring multiple copies of a 8.2-kb human *TSPY* DNA showed a spermatogonia/spermatocyte-specific expression of the transgene, similar to the human TSPY expression pattern [50]. Although the respective transgene expression could be influenced by the length of the promoter, integration sites, and genetic background of the hosts, it is conceivable that a combination of species-specific *cis* and *trans*-elements are important for the differential expression of the endogenous *hTSPY* and *rTspy* in respective types of germ cells.

During mammalian spermiogenesis, dynamic chromatin remodeling occurs in germ cell nuclei. Histones are replaced by protamines, and the chromatin is packed into highly condensed sperm nucleus [45,51]. Various studies suggest that certain factors (e.g. histone chaperones tNASP and CIA-II) are involved in the replacement of histones during spermiogenesis [51–53]. The general role of histone chaperones has been postulated to associate with histones and facilitate their interactions with other molecules without being components of the final reaction product and regulating histone metabolism [54]. NAP1, another histone chaperone, shares the TSPY/SET/NAP1 domain with rTspy, binds to the core histones H2A and H2B, and assembles nucleosome or modulates chromatin structure [33–35]. Further, SET/TAF-Iβ has been demonstrated to interact with sperm basic proteins and decondenses the sperm chromatin in the fertilized egg [55]. In the present study, we demonstrated that the rTspy protein was capable of binding core histones, H2A, H2B, H3, and H4, but preferentially in the cytoplasm of elongating spermatids (Fig. 2). Although its cytoplasmic location precludes it to have any role in nucleosome assembly, as ascribed for SET/TAF-Iβ, its interactions with the core histones at the period of spermiogenesis when the core histones are being replaced by protamines suggest that the rTspy could be an important chaperone for these sequestered core histones. We hypothesize that rTspy is involved in the metabolism of core histones, i.e. sequestrating the exported histones from nucleus and targeting them for degradation, in elongating spermatids.

The apparent decay of the rodent *Tspy* gene on their Y chromosome, i.e. gene inactivation in the laboratory mouse and spermatid-restricted expression in others, suggests that functions assigned to those of higher organisms, e.g. human and bovine, in germ-cell proliferation and meiotic division will most likely be performed by other genes in the rodent genomes, as illustrated in the human Y-located HSFY and autosomal HSFY-like system, discussed above. Conceivably, the autosomal *Tspy-like* (*Tspyl*) or other Y-located gene(s) would have assumed function(s) of TSPY in the rodent germ cell biology. Alternatively, other genes on the Y chromosome, e.g. *Sry* and *Eif2s3y*, have been postulated to serve vital function for spermatogenesis and are expressed as late as the round spermatid stage [23,25]. The *Eif2s3y* (also termed as *Eif2*γ) gene encodes a translation initiation factor and is essential for spermatogenic cells to proceed to the round spermatid stage in the mouse. The human homologous gene, EIF2γ, however, is not located on the Y chromosome [25,41], suggesting the reverse situation in which Y-located gene on the rodents is being replaced by an autosomal gene in humans. These, as well as numerous *Tspy-like* genes identified on the X chromosome and autosomes of the mouse, rat and human [26,27], might be capable of substituting the prescribed function(s) of TSPY in germ cells at different developmental stages. Interestingly, mutations of the human *TSPY-L1* gene on chromosome six have been demonstrated to be

responsible for a certain sudden infant death syndrome that is also associated with dysgenesis of the testes (SIDDT) [56]. The mutation introduced a stop codon upstream of the coding sequence for the TSPY/SET/NAP1 domain, thereby truncating an essential interactive domain common among members of this protein family. Patients with SIDDT show various abnormalities in the nervous system as well as testicular development, suggesting a likely role for TSPY-L1 in testicular function. Further studies on the relationship between the Y-located *TSPY*/*Tspy* and numerous autosomal and X-located *Tspy-like* genes will be significant in providing insights into this family of proteins, whose members are associated with each other primarily by their conserved cyclin B binding TSPY/SET/NAP1 domain.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.08.191.

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Original article

Testis-specific protein Y-encoded gene is expressed in early and late stages of gonadoblastoma and testicular carcinoma in situ☆

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Abstract

The testis-specific protein Y-encoded gene (TSPY) is a tandem repeat gene located at the critical region for the gonadoblastoma locus on Y chromosome that predisposes the dysgenetic gonads of intersex individuals to oncogenesis. The expression and molecular properties of TSPY suggest that it is the putative gene for the gonadoblastoma locus on Y chromosome. In this study, we examined the expression of TSPY and other germ cell tumor markers in 4 cases of gonadoblastoma using immunostaining techniques. Our results showed that TSPY expression was closely associated with initiation and various stages of gonadoblastoma development. TSPY protein localized with established germ cell tumor markers, such as the placental alkaline phosphatase, c-KIT, and OCT3/4, in the same tumor cells of both gonadoblastoma and adjacent carcinoma in situ, the precursor for germ cell tumors. These findings support the candidacy of TSPY as the gene for the gonadoblastoma locus on Y chromosome and suggest that TSPY could be a significant marker for these types of germ cell tumors. Published by Elsevier Inc.

Keywords: Y chromosome; Gonadoblastoma; Carcinoma in situ; TSPY; Gonadoblastoma locus on Y chromosome

1. Introduction

Gonadoblastomas are benign tumors consisting of aggregates of large round germ cells and immature Sertoli/granulosa cells surrounded by ovarian type stroma [1,2]. They occur most frequently (at a rate as high as 66%) in the dysgenetic gonads of intersex individuals who have either the whole or a part of the Y chromosome (e.g., 46,XY or 45,X/46,XY) in their genome [3]. Patients with Turner syndrome with a 45,X chromosome complement and residual Y chromosome material are also at risk for gonadoblastoma developing, although at a much lower rate (range 7% to 10%) [4–6]. These tumors are capable of synthesizing sex hormones, such as estrogen or testosterone, leading to either feminization or virilization of the dysgenetic gonads, respectively [3,5]. Approximately 80% of these intersex patients are phenotypic females, while 20% are males [3]. If left untreated, such confined tumors could develop into more aggressive ovarian dysgerminoma or testicular germ cell tumors [7,8].

In 1987, Page [9] proposed the existence of the gonadoblastoma locus on the Y chromosome to explain the high frequency of gonadoblastoma in the dysgenetic gonads of XY females. Gonadal dysgenesis is a complex disorder with a variable spectrum of phenotypes, and, in addition to mutations of the primary sex determining gene, SRY, may be caused either by a structural aberration of a gene involved in sex differentiation or, especially in the milder forms, as results of hormonal dysregulation during early gonadal development. The gonadoblastoma locus on Y chromosome gene(s) is postulated to serve a normal function in the testis but could predispose the dysgenetic gonads of intersex individuals to tumorigenesis [9,10]. Therefore, in the intersex/

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Table 1
Specimens from patients with gonadoblastoma

Patient number	Age (yrs)	Genotype	Phenotype	References
1	16	46,XY,SRY gene mutation, resulting in amino acid substitution Y127F at the high mobility group box	Female, primary amenorrhea, normal female external genitalia, fibrotic ovarian tissue with gonadoblastoma	[16]
2	17	46,XX/48,XXYY/47,XXY	Male, inguinal testis with gonadoblastoma, CIS and some ovarian stroma	[17,18]
3	14	45,X/46,XY	Female with Turner syndrome, streak gonads with gonadoblastoma	[17,18]
4	12	45,X/46,XY	Female with Turner syndrome, streak testis with gonadoblastoma	

sex-reversed patients, the presence of the Y-located gonadoblastoma locus on Y chromosome gene(s) could be considered as a gain of function(s) by the germ cells in a predominantly female environment. For the males, the SRY gene could be functional, while those downstream of this primary switch gene might be affected, resulting in streaked or dysgenetic gonads/testes. Based on these postulations, the gonadoblastoma locus on Y chromosome gene(s) could potentially be a protooncogene or tumor-promoting gene by a yet unknown mechanism.

Genetic mapping studies have narrowed the location of the gonadoblastoma locus on Y chromosome gene(s) to within 1–2 Mb of deoxyribonucleic acid (DNA) at deletion interval 3, proximal to the centromere on the short arm, and perhaps to a portion of interval 4 on the long arm [9,11,12]. Recent complete sequencing of the male-specific region Y DNA of the human Y chromosome has revealed the tandem repeat testis-specific protein Y-encoded gene (TSPY) gene cluster, encompassing approximately 0.7 MB of DNA, as the currently known functional genes within the gonadoblastoma locus on Y chromosome critical region [13]. Previous studies have shown that TSPY is indeed present and expressed in XY females and intersex individuals with gonadoblastoma [7,14,15].

Gonadoblastoma is a special form of germ cell tumor that resembles histologically that of the testicular carcinoma in situ (CIS) or intratubular germ cell neoplasia unclassified, the precursor for the testicular germ cell tumors. Although the expression of TSPY has been reported in clinical specimens of these types of early germ cell tumors, to our knowledge, the exact relationship of TSPY to other germ cell tumor markers (i.e., the placental/germ cell alkaline phosphatase, c-KIT [a type III tyrosine kinase receptor for stem cell factor], OCT3/4 [or POU5F1, a member of the POU family of transcription factors], and Ki-67 [a monoclonal antibody for proliferation marker]) has not been investigated in terms of the exact cellular locations and the tumorigenic development. We studied 4 cases of gonadoblastoma and showed the co-expression of TSPY, and these germ cell tumor markers, in the tumor cells of these specimens. Furthermore, using the TSPY as a marker, we were able to trace the probable oncogenic sequence of one case of gonadoblastoma, and could show the parallel TSPY expression patterns in both gonadoblastoma and CIS in another case.

2. Materials and methods

2.1. Patients

Intersex patients and those with Turner syndrome with a whole/part of Y chromosome (Table 1) were initially identified in endocrine and genetics clinics at the University of California, Los Angeles (patient No. 1) and San Francisco (patient No. 4), and University Hospital Copenhagen (patient Nos. 2 and 3). The clinical phenotypes and genotypes of these patients have been reported previously [16–18]. All studies of human archival pathologic specimens were performed under an exempt protocol approved by the Institutional Committee on Human Research, VA Medical Center, San Francisco.

2.2. Antibodies

Several monoclonal and polyclonal antibodies were used in this study. A monoclonal antibody (SF-7) against the human TSPY was generated in our laboratory [19]. Other commercially available antibodies against various germ cell tumor markers (i.e., rabbit polyclonal antibodies against placental/germ cell alkaline phosphatase [SP15, RM-9115; Lab Vision, Fremont, CA] and c-KIT [A4502; Dako, Carpinteria, CA], a polyclonal goat anti-Oct3/4 antibody [C-20, sc-8629; Santa Cruz Biotechnology Inc. Santa Cruz, CA] and a rabbit monoclonal antibody against Ki-67]Clone SP6, RM-9106; Lab Vision]) were purchased from the respective vendors.

2.3. Immunohistochemistry and immunofluorescence

Immunostaining was performed with standard methods using specific antibodies, as described previously [7,19]. Five-micron tissue sections were generated with a microtome from archival pathologic preparations, deparaffinized, and treated with an antigen retrieval procedure (50 mM Tris-HCl, pH10 at 95°C for 30 minutes). The slides

were incubated in 3% H_2O_2 solution for 15 minutes to inactivate the endogenous peroxidase, blocked with 3% bovine serum albumin, 0.1% TritonX-100 in phosphate-buffered saline for 1 hour, and reacted overnight at 4°C with respective primary antibody. The antibody dilutions were TSPY at 1:2000, placental/germ cell alkaline phosphatase at 1:50, c-KIT at 1:300, OCT3/4 at 1:300, and Ki-67 at 1:100 ratios. After extensive washing with phosphate-buffered saline, the slides were incubated with respective secondary antibodies as: a biotinylated anti-mouse immunoglobulin G (IgG) (1:500) for TSPY; biotinylated anti-rabbit IgG (1:500) for placental/germ cell alkaline phosphatase, c-KIT, and Ki-67; and a biotinylated anti-goat IgG (1:500) for OCT3/4. Various substrates for horseradish peroxidase were used to visualize the immunoreactions (i.e., with diaminobenzidine tetrahydrochloride for TSPY, diaminobenzidine tetrahydrochloride + Ni for Ki-67, alkaline phosphatase red for placental/germ cell alkaline phosphatase, and blue for c-KIT). The slides were counterstained with hematoxylin or fast red nuclear stain, examined, and recorded with a Zeiss Axiophot microscope (Carl Zeiss, Inc., Thornwood, NY).

For immunofluorescence, fluorescein isothiocyanate-conjugated anti-mouse IgG (for TSPY), Texas Red-conjugated anti-goat (for OCT3/4) and Alexa 594-conjugated anti-rabbit IgG (for placental/germ cell alkaline phosphatase, c-KIT, and Ki-67) were used to visualize the primary antibody reactions. The immunofluorescence was examined with a Leica TCS SP confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) and recorded with a digital camera [20].

Normal adult human testis sections were used as positive controls for TSPY. Testicular seminoma specimens were used as positive controls for TSPY, placental/germ cell alkaline phosphatase, c-KIT, and OCT3/4. Sections immunostained with the same procedure, but without the corresponding primary antibody, were used as negative controls.

3. Results

To establish the TSPY as a candidate gene for the gonadoblastoma locus on Y chromosome, we had used immunostaining techniques to examine the expression of TSPY in 4 cases of gonadoblastoma (Table 1) with reference to various germ cell tumor markers (i.e., placental/germ cell alkaline phosphatase, c-KIT, and OCT3/4), which are preferentially expressed in testicular germ cell tumors [8,21]. Placental/germ cell alkaline phosphatase, c-KIT, and OCT3/4 are expressed in early male germ cells during embryogenesis but are down regulated during the late stages of gestation [21]. They are reactivated postnatally upon germ cell oncogenesis and, thus, are markers for testicular germ cell tumors. Previous studies showed that TSPY is also expressed in similar stages of embryogenesis, but its expression persists throughout the testicular maturation, reaching a maximal level in spermatogonia in normal adult testis [21,22]. In this study, despite their phenotypic heterogeneity, all 4 cases of gonadoblastoma were positive for TSPY and the aforementioned tumor markers. Some of these cases were included in previous studies [16,18,23,24], but, to our knowledge, TSPY had not been investigated. Two cases, in particular, were significantly informative and are illustrated in detail later.

The first specimen was derived from an XY female (Table 1, patient No. 1) who had an A->T transversion mutation at codon 127 of her SRY gene on a cytogenetically normal Y chromosome [16]. Initial pathologic examination of her dysgenetic gonads showed nests of tumor cells composed of germ cells and sex cord elements. Some tumor nests showed prominent signs of calcification [16]. Histologically, many of these tumor nests resembled atretic follicles and young corpus luteum, many of which were intensively positive for TSPY protein (Fig. 1A). At high magnification, these follicular structures were apparently void of any cellular structures (Fig. 1B). One or more TSPY positive cells initially appeared at the peripheral region of the empty structures (Fig. 1C). These cells showed proliferative growth and eventually filled up the entire follicular space, resulting in nests of tumor cells (Fig. 1D–H). This confined tumorigenic growth could either continue to expand or merge with adjacent structures, leading to large nests of tumor cells, characteristic of a gonadoblastoma. Most TSPY positive cells (Fig. 1I, L, O, and R) were also positive for placental/germ cell alkaline phosphatase (Fig. 1J), c-KIT (Fig. 1M), OCT3/4 (Fig. 1P), and Ki-67 (Fig. 1S), respectively. On merged images, TSPY was co-expressed with the respective tumor markers in most, but not all, tumor germ cells (Fig. 1K, N, Q, and T), despite variation in subcellular locations. TSPY protein was located in both cytoplasm and nuclei of the tumor cells. Placental/germ cell alkaline phosphatase and c-KIT were primarily located on the cell surface, and OCT3/4 and Ki-67 were located in the nucleus. The immunostaining showed some degrees of heterogeneity among individual cells, which is in concert with previous observations [25]. Primarily, TSPY expression was tightly linked to the expression of placental/germ cell alkaline phosphatase, c-KIT, and OCT3/4, but to a lesser extent with that of Ki-67. The co-expression of TSPY with Ki-67 suggests that at least some of these TSPY positive cells possessed proliferative properties.

Numerous studies have suggested that gonadoblastoma and testicular CIS or intratubular germ cell neoplasia unclassified, the precursor for testicular germ cell tumors, share common etiologies. Although both gonadoblastoma and CIS might be present in the same individuals [24–27] and TSPY could be present in CIS cells in adult men [28], the simultaneous detection of TSPY in both gonadoblastoma and CIS on the same specimen has not been shown with reference to other germ cell tumor markers. We examined a second informative case of gonadoblastoma from a dysgenetic testis with both CIS and gonadoblastoma of a mosaic 46,XX/48,XXYY/47,XXY male individual (Table

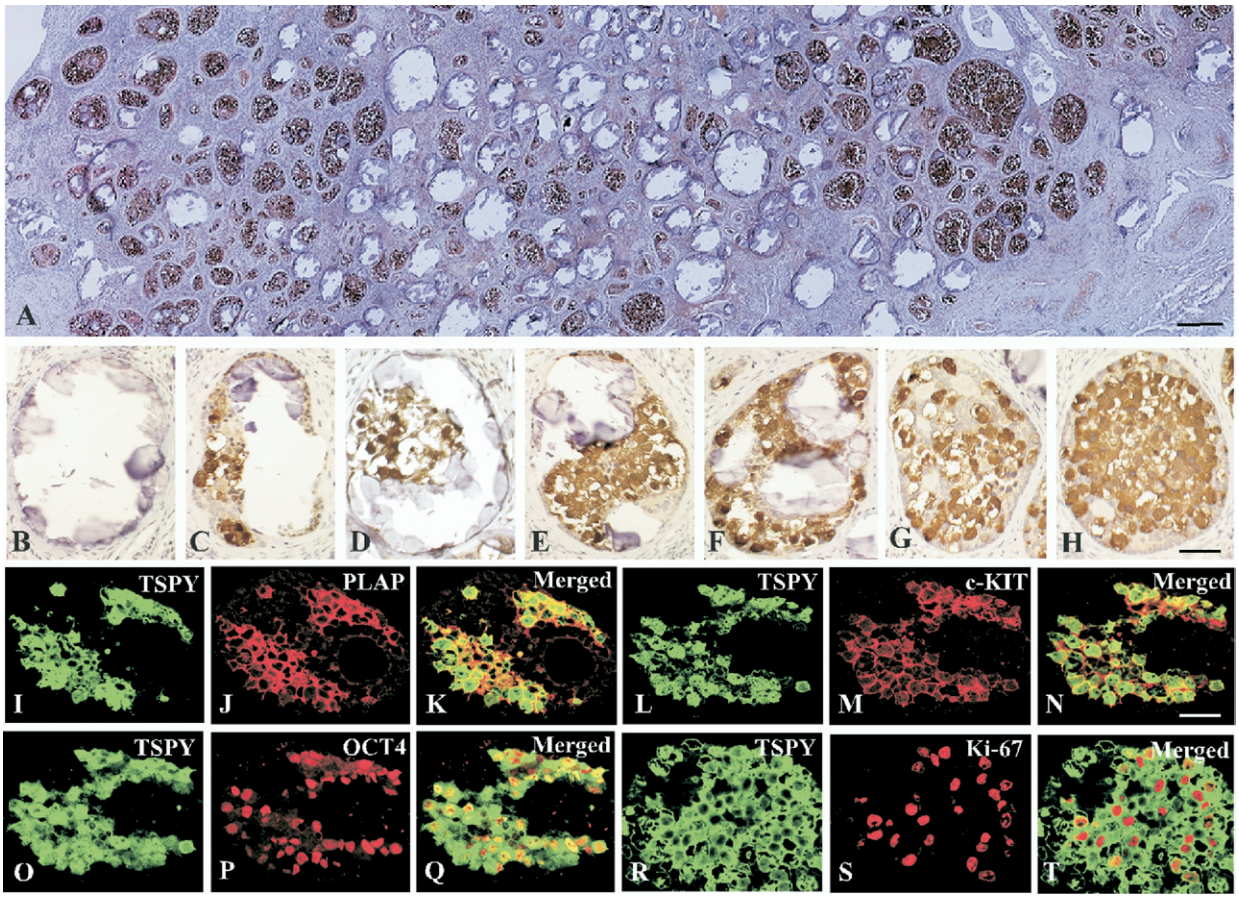

Fig. 1. Co-expression of TSPY and germ cell tumor markers in the gonadoblastoma of a XY sex-reversed patient. (A) An expanded view of the gonadoblastoma specimen that was immunostained with a TSPY monoclonal antibody showing the distribution of TSPY protein in nests of tumor germ cells, but not the stroma of the dysgenetic gonad. (B–H) Various follicle-like structures showing various degrees of tumorigenesis. TSPY-positive cells were initially observed along the periphery that took on a proliferative growth (D–G) and eventually filled up the entire structure (G–H). Immunofluorescence showed that most of the TSPY expressing cells ([I], [L], [O], and [R]) were also positive for numerous germ cell tumor markers, including placental/germ cell alkaline phosphatase (J), c-KIT (M), and OCT3/4 (P), as well as the proliferative cell marker ([S] Ki-67). The merged images ([K], [N], [Q], and [T]) showed that TSPY was co-expressed with these tumor germ cell markers, despite their variation in subcellular locations and heterogeneity in the staining intensities. Bars represent 200 μm in (A); 50 μm in (B)–(T).

1, patient No. 2). Histologically, the dysgenetic testis showed components of both CIS and gonadoblastoma (Fig. 2A). The gonadoblastoma portion showed the characteristics of nests of large germ cells with prominent nuclei, Sertoli/granulosa somatic cells, and fluid-filled Call-Exner bodies (Fig. 2B, arrows). Although there could be heterogeneity in staining intensity, both mature (Fig. 2C and D, green arrows) and immature (Fig. 2C, blue arrow) germ cells were positive for TSPY. The CIS portion showed the typical peripheral location of the malignant germ cells (Fig. 2H) that were positive for the TSPY antibody (Fig. 2I and J). The TSPY staining was most prevalent on the nuclei of the tumor germ cells (Fig. 2D and J). Again, immunostaining for placental/germ cell alkaline phosphatase (Fig. 2E and K, red signals), c-KIT (Fig. 2F and L, blue signals), and OCT3/4 (Fig. 2G and M, brown signals) showed the presence of these germ cell tumor markers in both gonadoblastoma and CIS (Fig. 2E–G for gonadoblastoma and Fig. 2K–M for CIS).

4. Discussion

The human Y chromosome is a relatively gene-poor portion of the human genome. There are only 3 major loci mapped on this chromosome, namely sex determination, infertility, and gonadoblastoma predisposition [10,13]. Various studies have clearly established the SRY to be the testis-determining factor, responsible for switching on the male sex determination [29–31]. The genes for both infertility and the gonadoblastoma locus on Y chromosome have been isolated [10,32] but have not been established by functional methods. As a candidate for the gonadoblastoma locus on Y chromosome, TSPY has been shown to express in limited cases of gonadoblastoma tissues; however, its relationship to the other germ cell tumor markers has not been clearly established. In this study, we have showed that TSPY was positive in all 4 cases of gonadoblastoma examined. Significantly, we were able to show the probable sequence of tumorigenesis in the dysgenetic gonads in in-

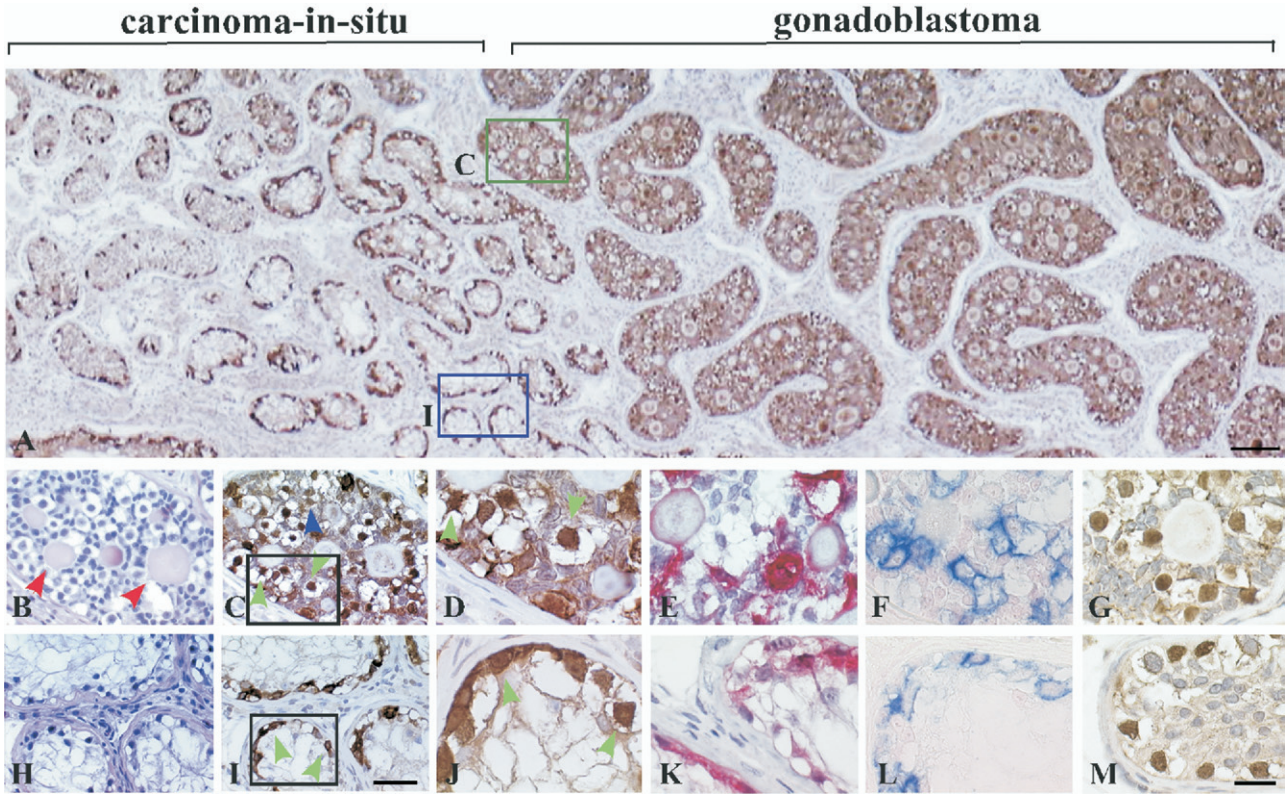

Fig. 2. Co-expression of TSPY and germ cell tumor markers in tumor cells of a 46,XX/48,XXYY/47,XXY patient with both CIS and gonadoblastoma in his dysgenetic testis. (A) An overview of the testicular neoplasia with both CIS (left) and gonadoblastoma (right). (B and H) Hematoxylin-eosin staining of the adjacent section of the green and blue boxed areas representing the gonadoblastoma (C) and CIS (I) regions in (A), respectively. (D and J) Enlarged views of boxed areas in (C) and (I), respectively. Placental/germ cell alkaline phosphatase, c-KIT, and OCT3/4 were represented in (E), (F), and (G) for gonadoblastoma and (K), (L), and (M) for CIS on adjacent sections, respectively. Different color substrates from Vector Laboratories (Burlingame, CA) (red for placental/germ cell alkaline phosphatase, blue for c-KIT, and brown for OCT3/4) were used for visualization of the corresponding primary antibodies and observed under a Zeiss Axiophot microscope. Although there could be differences in intensities, both immature and mature germ cells stained positively with both TSPY (C) and OCT3/4 (G) at their nuclei, while placental/germ cell alkaline phosphatase (red) and c-KIT (blue) were primarily located at the surface of malignant germ cells. Bars represent 100 μm in (A), 50 μm in (B), (C), (H), and (I); and 20 μm in (D)–(G) and (J)–(M).

dividuals with sex chromosome aneuploidy, and the similarities in expression patterns between TSPY and other germ cell tumor markers in both gonadoblastoma and CIS on the same specimens. Our results clearly support TSPY as a tumor germ cell marker for both gonadoblastoma and CIS. Its genetic location within the critical region of the gonadoblastoma locus on Y chromosome locus strongly suggests that TSPY is the putative gene for this oncogenic locus on the Y chromosome.

TSPY encodes several slightly polymorphic proteins [33] harboring a domain pertaining to a protein superfamily, represented by the SET oncoprotein and nucleosome assembly protein 1 [34–37]. Numerous members of this family bind the type B cyclin and are involved in cell cycle regulation [37,38]. Therefore, TSPY potentially could be involved in certain regulatory aspects of the cell cycle. Indeed, TSPY has been hypothesized to play such normal roles in both mitotic proliferation and meiotic division of male germ cells [10]. When it is ectopically expressed, TSPY could affect cell cycle dysregulation and exert proliferative effects on susceptible cells, thereby predisposing them to oncogenesis. Recently, Kido and Lau [19] showed that a 2.4-kb TSPY promoter was capable of mediating an expression of a reporter gene in the secondary follicles in the ovaries of female transgenic mice. These findings further support the functionality of TSPY in a female and/or ovarian environment, a necessary step in the initiation of the gonadoblastoma oncogenic process in XY sex-reversed individuals. Although the exact mechanism(s) by which TSPY exerts its oncogenic effects in germ cell tumors still must be defined experimentally, the present observations further substantiate the role of TSPY in gonadoblastoma.

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Original contribution

The Y-encoded TSPY protein: a significant marker potentially plays a role in the pathogenesis of testicular germ cell tumors☆

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Testicular germ cell tumors

Summary The testis-specific protein Y-encoded (TSPY) gene is the putative gene for the gonadoblastoma locus on the Y chromosome (GBY) that predisposes dysgenetic gonads of intersex patients to gonadoblastoma development. TSPY is expressed at high levels in gonadoblastoma tissues, supporting its possible oncogenic function in this type of germ cell tumors. To explore the possibility that this Y chromosome gene is also involved in pathogenesis of the more common testicular germ cell tumors (TGCTs), we have conducted various expression studies using immunohistochemistry, Western blotting, and reverse transcription-polymerase chain reaction analysis on 171 cases of TGCTs and selected normal testis controls. Our results demonstrated that TSPY protein is abundantly expressed in the precursor, carcinoma in situ or intratubular germ cell neoplasia unclassified, and seminoma, but only minimally or not expressed in various types of nonseminomas. TSPY coexpresses with established germ cell tumor markers (such as placental-like alkaline phosphatase, c-KIT, OCT4) and proliferative markers (such as Ki-67 and cyclin B1) in the same tumor cells at both RNA and protein levels. Ectopic TSPY expression in cultured cells up-regulates progrowth genes, including those at chromosome 12p13, frequently gained/amplified in TGCTs. Our results suggest that TSPY, in combination with other markers, could be an important marker for diagnosis and subclassification of TGCTs and support its role in the pathogenesis of both gonadoblastoma and TGCTs.

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1. Introduction

The testis-specific protein Y-encoded (TSPY) gene is a tandemly repeated gene on the short arm of the human Y chromosome [1-3]. Most of its functional transcriptional units have been mapped within the critical region harboring the gonadoblastoma locus on the Y chromosome (GBY) [4-6], the only oncogenic locus on this male-specific chromosome. It is hypothesized to serve a normal physiologic function in germ cell proliferation and/or differentiation, but could predispose incompatible germ cells, for example, those in an ovarian environment or dysfunctional/dysgenetic testis, to tumorigenesis [4,7]. Gonadoblastoma most frequently arises in dysgenetic gonads of XY women, intersex individuals, and—to a lesser extent—Turner patients with residual Y chromosome materials [8-12]. Recent studies demonstrated that TSPY sequences are indeed present in the genomes of these XY women and intersex individuals, and are abundantly expressed in this special type of germ cell tumors [13-15], thereby supporting the candidacy of TSPY as the gene for GBY.

Several studies have documented the expression of TSPY in the more common forms of testicular germ cell tumors (TGCTs) of adult testis, classified as seminomas and nonseminomas [2,7,16-18]. However, its value as a diagnostic marker for subtypes of these prevalent cancers among young men between 15 and 35 years of age has not been established. At present, a detailed investigation regarding its colocalization with other known markers has not been performed so far. Furthermore, various isoforms of TSPY transcripts and proteins have been demonstrated in cancerous samples [16]. It is uncertain if such isoforms exist in various types of TGCTs. To address these questions, we conducted a comprehensive study in establishing its expression pattern with reference to those of other germ cell tumor markers, such as OCT4, c-KIT, and placental-like alkaline phosphatase (PLAP), alpha fetal protein (AFP), and human chorionic gonadotrophin (hCG) [19]. Our results showed that TSPY is predominantly expressed in testicular seminoma and the precursor, carcinoma in situ (CIS) or intratubular germ cell neoplasia unclassified (ITGCNU), but not in various types of nonseminomas, including embryonal carcinoma, teratoma, choriocarcinoma, and yolk sac tumors. Its expression pattern parallels those of OCT4, c-KIT, and PLAP in CIS/ITGCNU and seminomas, but not AFP and hCG in nonseminomas. The differential expression pattern of TSPY in seminomatous and nonseminomatous germ cell tumors suggests that it can be used as a diagnostic marker for detection of precursors of germ cell tumors and for subtyping of TGCTs. Hence, TSPY, in combination with other markers, could be an important marker for diagnosis and subclassification of TGCTs.

Testicular germ cell tumors are postulated to originate from the CIS/ITGCNU precursor. Currently, the mechanism(s) by which this premalignant precursor initiates and develops into both seminomas and nonseminomas is unknown. Various genetic studies have demonstrated that CIS/ITGCNU and TGCTs are aneuploid. A gain of complete short arm of chromosome 12, and sometimes amplification of certain portion of it, has been the consistent change(s) in the evolving germ cell tumor genome [19-22]. Such gain of chromosome 12p genes seems to be associated with advancement of the oncogenic process(es) and increase in pluripotency of the tumor cells [20,21,23,24]. Previously, we demonstrated that ectopic TSPY expression in cultured cells up-regulates progrowth genes, including those at chromosome 12p13, and accelerates the G_2/M transition in the cell cycle [25]. Analysis of available microarray data demonstrates a correlation between TSPY expression and up-regulation of certain chromosome 12p13 genes in clinical CIS/ITGCNU and TGCT samples [26,27]. The cell cycle regulatory function(s) of TSPY and the present results, therefore, support a role(s) for this Y chromosome gene in the pathogenesis of both gonadoblastoma and TGCTs.

2. Materials and methods

2.1. Tissue collection

A total of 171 TGCTs consisting of 86 seminomas, 85 nonseminomas, and 17 normal testicular tissues were analyzed in the present study. They were obtained from Department of Urology, Hirosaki University School of Medicine, Cooperative Human Tissue Network (CHTN), and Department of Pathology, Veterans Affairs Medical Center, San Francisco. Except for 2 cases of nonseminomas that occurred at ages of 4 and 9 years, most TGCT patients were between 19 and 68 years of age. A total of 131 cases with clinical staging information from the Department of Urology, Hirosaki University, Japan, were used in correlating the TSPY expression with clinical staging using a crude scoring system, as described in Results. Of these 131 cases, 61 were seminomas and 70 were nonseminomas consisting of embryonal carcinoma, yolk sac tumor, teratoma, choriocarcinoma, and mixtures thereof. The median ages for the seminoma and nonseminoma groups were 34 and 27.5 years, respectively. Patients with mixed germ cell tumors harboring nonseminomatous and seminomatous components were considered nonseminomas. The tumor type classification was selectively examined and confirmed by a pathologist at the VA Medical Center. The seminoma and CIS/ITGCNU components were excluded in these nonseminoma specimens and were studied separately in our scoring analysis. Frozen samples of TGCTs were obtained from the CHTN and used for protein and RNA analyses. The classification of these frozen samples was based on pathologists' reports provided from the CHTN. Additional protein and RNA samples from various TGCTs were provided by Professor Leendert Looijenga, Erasmus Medical Center, Rotterdam, the Netherlands. These were previously characterized by immunostaining with specific TSPY antibody. The presence of TSPY RNA and proteins in these

samples corresponded to the immunohistochemistry results. Tissue arrays containing normal fetal and adult testes were purchased from Cybrdi Inc (Frederick, MD). All studies on human archival pathologic specimens were performed under a protocol approved by the institutional committee on human research, VA Medical Center, San Francisco.

2.2. Antibodies

The mouse monoclonal antibodies (nos. 2 and 7) against recombinant TSPY were generated in our laboratory [28]. Other antibodies were obtained from the following commercial sources: (1) Lab Vision (Fremont, CA), a rabbit polyclonal antibody against PLAP (SP15, RM-9115), a rabbit monoclonal antibody against Ki-67 (Clone SP6, RM-9106), a rabbit antibody against the AFP (RB9064), a mouse monoclonal antibody against the hCG (SPM105); (2) Dako Corp (Carpinteria, CA), a rabbit polyclonal antibody against c-KIT and a mouse monoclonal antibody against human cyclin B1 (A4502); (3) Sigma Biochemicals (St. Louis, MO), a mouse monoclonal antibody against tubulin; and (4) Santa Cruz Biotechnology Inc (Santa Cruz, CA), a goat polyclonal antibody against OCT4 (C-20, sc-8629). The OCT4 antibody has been demonstrated to be informative for immunohistochemistry and Western blotting of TGCTs [29].

2.3. Immunohistochemistry and immunofluorescence

Immunohistochemical staining was performed as previously described [28]. Briefly, formalin-fixed and paraffin-embedded TGCT tissue sections were deparaffinized with xylene, and hydrated with an ethanol-water series. Antigen retrieval was performed by incubating the slides in 50 mmol/L Tris-HCl (pH 10) at 95°C for 20 to 30 minutes. Endogenous peroxidase was inactivated in 3% H_2O_2 for 15 minutes at room temperature. The sections were blocked with 3% BSA, 0.1% Triton X-100 in PBS for 1 hour, and were incubated overnight at 4 °C with the respective primary antibodies, at dilution ratios of 1:1000 for TPSY monoclonal antibody, 1:50 for PLAP rabbit antibody, 1:500 for c-KIT rabbit antibody, 1:500 for OCT4 goat antibody, 1:200 for AFP antibody, 1:50 for hCG antibody, and 1:100 for Ki-67 rabbit monoclonal antibody. The primary antibodies were then detected with corresponding biotinylated secondary antibodies and visualized via avidin-biotin detection and substrate kits (Vector Laboratories, Burlingame, CA). The immunostained sections were reviewed independently by at least 2 investigators. For immunofluorescence double staining, sections were processed as described earlier, the signals were visualized with FITC or Alexas 594 conjugated secondary antibodies (Cell Signaling, Danvers, MA) and fluorescence microscopy. Normal testis sections were used as positive control for TSPY. As negative controls, parallel sections were similarly processed without the respective primary antibody for each immunostaining experiment.

2.4. Reverse transcription-polymerase chain reaction analysis

Frozen TGCT tissues were obtained from the CHTN. Total RNAs were isolated from these frozen tissues by using TRIzol reagents (Invitrogen, Carlsbad, CA). After treatment with RNase-free DNase (Promega, Madison, WI), 2 μg of treated total RNA were used for cDNA synthesis with SuperScripts III Kit (Invitrogen) in a 20-μL reverse transcriptase reaction mixture. Polymerase chain reaction (PCR) was performed with 1 μL of each of the cDNA reactions with specific primer sets and a touchdown protocol [16]. Semiquantitative reverse transcription (RT)-PCR and estimation of amplified products were performed as previously described [25]. GAPDH and HPRT were used as controls in respective experiments, as noted.

2.5. Western blot analyses

Western blotting was performed with established procedures [28,30]. Frozen tissues were homogenized in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L ethylene glycol tetraacetic acid, 1% Triton X-100) with a polytron, and centrifuged at 16000 g for 10 minutes. The protein concentration was determined by a Bio-Rad DC protein assay kit (Bio-Rad, Richmond, CA). One hundred micrograms of each lysate was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and transferred onto nitrocellulose membranes via electroblotting. Human HEK293 cells were individually transfected with expression vectors for the cDNAs of various TSPY isoforms under the CMV promoter and analyzed similarly after 48 hours by Western blotting [28,30]. The membranes were incubated with various primary antibodies at various dilutions, as described earlier. The signals were visualized with respective horseradish peroxidase-conjugated secondary antibodies and ECL plus chemiluminescence kit (GE Healthcare, Piscataway, NJ). The filters were stripped and reprobed or parallel ones were probed with additional antibodies and the signals detected similarly.

2.6. Hierarchical clustering of gene expression data

Gene expression data of selected number genes mapped to chromosome 12 band p13 were obtained from the Gene Expression Omnibus (GEO) database [26]. The GEO data sets were as follows: normal testes (NT1, GSM31729; NT2, GSM31728; NT3, GSM31803), CIS/ITGCNU samples (ITGCNU1, GSM33594; ITGCNU2, GSM31730; ITGCNU3, GSM31731), seminomas (SE1, GSM33595; SE2, GSM31732; SE3, GSM33942), and embryonal carcinoma (EC1, GSM33944). Other microarray data sets were derived from published results [27]. The expression data in $\log_2$ ratios were analyzed by a 1-sample t test to detect significant differences in gene expression. Genes that

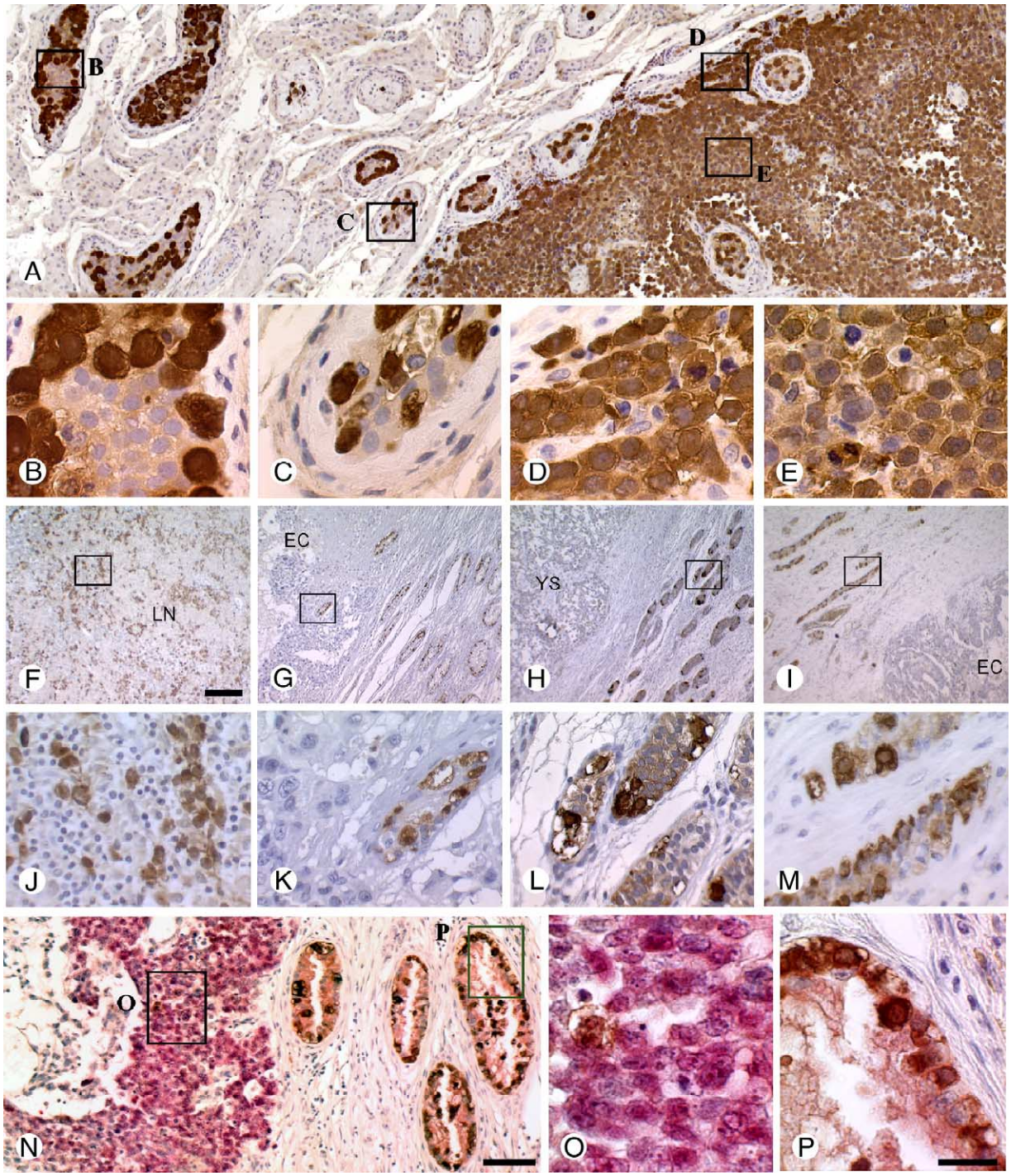

Fig. 1 Preferential TSPY expression in testicular seminoma and its precursor, CIS/ITGCNU. (A) An example of intense TSPY immunostaining of seminoma (right) harboring adjacent CIS/ITGCNU cells (left) in a 34-year-old patient. (B-E) Enlarged views of boxed areas in A showing CIS/ITGCNU (B, C) and seminoma (D, E) components. Immunostaining of TSPY on (F) a case of metastatic seminoma in lymph node, and (G-I) selected nonseminomas harboring adjacent CIS/ITGCNU cells. TSPY was intensely positive for metastatic seminoma cells (J) and CIS/ITGCNU cells but not the respective nonseminomas (K-M). (N) Double immunostaining of TSPY and OCT4 on an embryonal carcinoma (left) with adjacent CIS/ITGCNU (right) of a 35-year-old patient. (O-P) Enlarged views of corresponding boxed areas in N. LN indicates lymph node; YS, yolk sac tumor; EC, embryonal carcinoma. Bar in F represents 400 μm in F-I; bar in N represents 100 μm in A, J-N; and bar in P represents 20 μm in B-E and O-P.

showed differential expression with P values $\leq$ of .01 were considered statistically significant. Normalized gene expression ratios between samples were analyzed with significant analyses for microarray algorithm with less than 5% false discovery rate. The expression profiles resulting from this analysis were grouped based on similarity in pattern of their

expression by using hierarchical cluster analysis based on the Pearson correlation by TIGR MultiExperiment Viewer software version 4.01 [31].

3. Results

3.1. TSPY is preferentially expressed in seminomas and germ cell tumor precursors

To correlate the TSPY expression at the early stage of TGCTs, we examined a total of 171 cases of TGCTs harboring various features, including CIS/ITGCN, seminoma, yolk sac tumor, embryonal carcinoma, and choriocarcinoma, using immunostaining techniques. Our results showed that TSPY was preferentially expressed at high levels in CIS/ITGCNU and seminoma specimens (Fig. 1A-E). Such immunostaining persisted in metastatic seminoma cells in the lymph node (Fig. 1F, J). However, immunostaining signals for TSPY were at minimal or negative levels in nonseminomas, including teratoma, yolk sac tumors (Fig. 1H), and embryonal carcinomas (Fig. 1G, I, N, and O). Selected areas of the yolk sac tumors and embryonal carcinomas might contain clusters of CIS/ITGCNU whose tumor cells are highly positive for TSPY (Fig. 1G-I, K-M, N-P), as those in seminoma samples (Fig. 1B, C).

3.2. TSPY is coexpressed with established tumor markers for seminoma and CIS/ITGCNU

The preferential expression of TSPY in testicular seminoma and CIS/ITGCNU suggests that this GBY candidate gene could serve as a specific tumor marker for these types of germ cell tumors. To determine its expression pattern in reference to other established germ cell tumor markers, such as PLAP, OCT4, c-KIT, and the proliferative marker, Ki-67, we performed double immunofluorescence analysis on selected testicular seminoma and CIS/ITGCNU specimens. Our results demonstrated that TSPY was coexpressed in the same tumor germ cells of both types of TGCTs, despite variation of their subcellular locations and heterogeneity in staining intensity (Fig. 2). TSPY was located in both cytoplasm and nuclei of the tumor germ cells. Placental-like alkaline phosphatase and c-KIT (the tyrosine kinase receptor for stem cell factor) were primarily located on the cell surface, whereas OCT4 (the stem cell transcription factor) and Ki-67 (the proliferative marker) were located on the

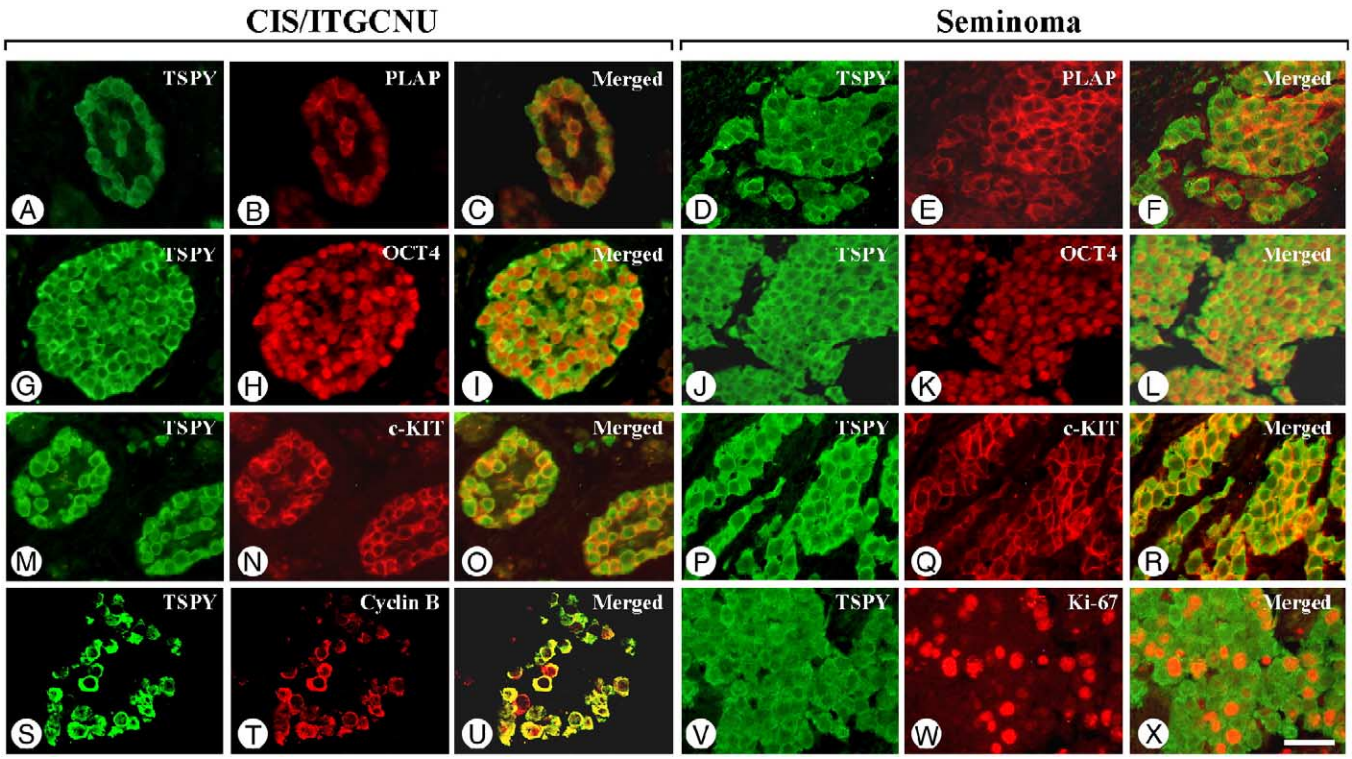

Fig. 2 Coexpression of TSPY and various tumor markers in CIS/ITGCNU (left 3 columns) and seminoma (right 3 columns) tumor germ cells. Double immunofluorescence of TSPY (green in A, D, G, J, M, P) and PLAP (red in B, E), OCT4 (red in H, K), and c-KIT (red in N, Q) showed that these tumor markers were expressed in the same tumor germ cells, as indicated in merged images (yellow-orange in C, F, I, L, O and R). TSPY (green in S and V) was coexpressed with cyclin B1 (a potential interactive partner, red in T) and the Ki-67 proliferative marker (red in W) in the same tumor germ cells, as revealed in respective merged images (yellow-orange in U and X). These specimens were derived from patients who were between 27 and 56 years old. Bar in X represents 20 μm in all images.

nuclei [32]. Similar to TSPY, cyclin B1 (the mitotic cyclin) could be located on both cytoplasm and nuclei. All other germ cell tumor markers coexpressed mostly with TSPY protein in the same tumor germ cells, as revealed by merged images of the respective tissue sections (Fig. 2C, F, I, L, O, R, and U), except the proliferative marker (Ki-67) that was only highly expressed in selected TSPY positive cells (Fig. 2X).

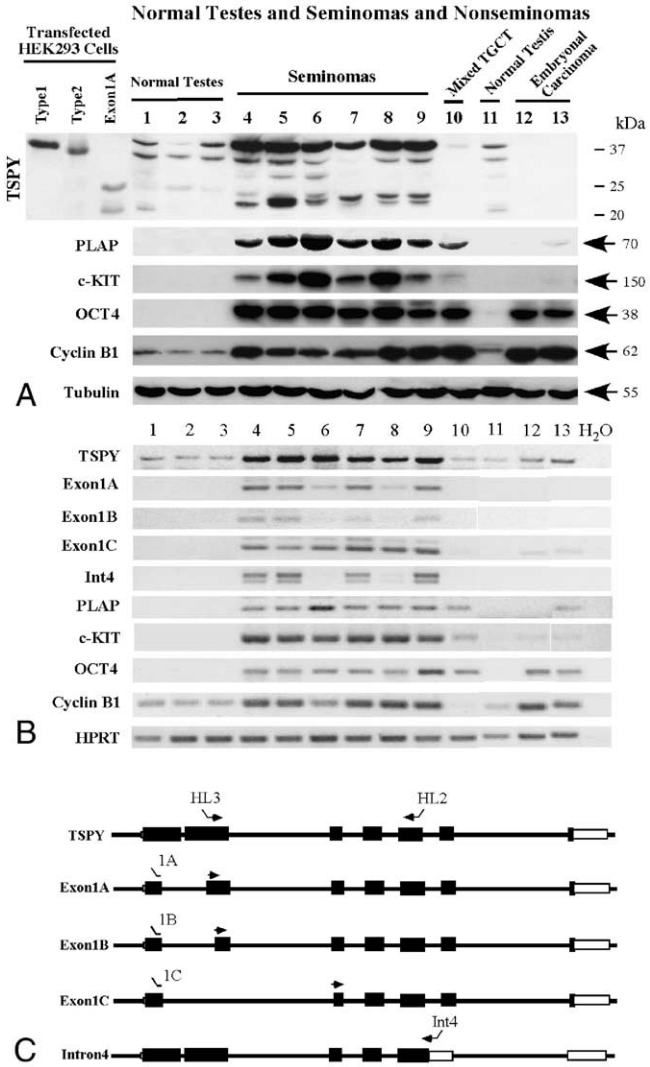

Fig. 3 Protein and RNA analyses of gene expression in normal testes, seminoma, and nonseminoma specimens. (A) Western blot analyses of various samples with a TSPY antibody showed multiple reactive protein bands, representing different isoforms of TSPY in protein lysates of normal testes (lanes 1-3, 11), seminomas (lanes 4-9), and nonseminomas (lanes 10, 12, 13). Some of these TSPY bands corresponded to those from HEK293 cells transfected with DNA plasmids expressing various TSPY isoforms (left 3 lanes, types 1 and 2 = full-length cDNA; Exon1A = cDNA of alternatively spliced transcript). The amounts of TSPY proteins were highest among the seminoma specimens, low but detectable levels in normal testes, and minimal amounts or none among the nonseminomas. Reprobing of the same or parallel filters showed similar expression patterns for PLAP, c-KIT, and—to a certain extent—OCT4 and cyclin B1. The latter markers also expressed at high levels in selected nonseminoma specimens (eg, mixed germ cell tumor in lane 10 and embryonal carcinomas in lanes 12 and 13). The relative amounts of tubulin seemed to be quite even among all samples. (B) Reverse transcription-polymerase chain reaction analyses of transcripts for TSPY isoforms (TSPY = total transcripts; Exon1A, Exon1B, Exon1C, and Int4 = alternatively spliced transcripts), and germ cell tumor markers (PLAP, c-KIT, OCT4, and cyclin B1) in the same samples used in Western blotting in A. Successful amplification of RT-PCR products with primer sets specific for the transcripts of the respective isoforms suggested that these variant TSPYs were present in the corresponding samples, particularly the seminomas (lanes 4-9). Although semiquantitative in nature, the amounts of the RT-PCR products paralleled the intensities of Western blot signals (detected in A), whereas the same analysis of a reference gene (HPRT) showed relatively even amounts of RT-PCR products among all samples. All RT-PCR images were obtained from negative printing of the respective ethidium bromide staining of the agarose gels. (C) Diagrammatic illustration of major variant transcripts originated from the expression of the TSPY tandem arrays on the human Y chromosome. Arrows indicate the positions of respective primers in the structural gene used to detect these variant transcripts by RT-PCR analysis (B). Solid and open boxes represent coding and noncoding sequences of the variant transcripts.

3.3. Variant TSPY proteins are expressed in both normal and tumor germ cells

To confirm our immunostaining results, selected cases of seminomas, nonseminomas, and normal testes were analyzed with Western blotting (Fig. 3A). Our results showed that TSPY was indeed expressed at high levels in seminoma samples, but minimally in mixed TGCT, and embryonal carcinomas. Normal testes showed reduced but detectable levels (Fig. 3A, lanes 1-3, 11). Interestingly, multiple bands were observed in both seminomas and normal testis samples (eg, Fig. 3A, lanes 1-9). These bands correspond to those expressed in HEK293 cells transfected with expression vectors for respective isoforms of TSPY proteins (Fig. 3A, left 3 lanes) [16]. Reprobing of the same filters with various specific antibodies showed that specific TGCT markers, such as PLAP, c-KIT, and OCT4, were highly expressed in seminoma samples, but not in normal testes. Significantly, both OCT4 and Cyclin B1 showed similar high levels of expression in the embryonal carcinoma samples as those of the seminomas. We also observed similar immunostaining intensity for these 2 markers in the same specimens.

Various TSPY isoforms are generated by alternative splicing events of transcripts originating from the same transcriptional units [16,33]. In general, these variant transcripts can be classified into 2 categories. The first one uses a cryptic donor site at codon no. 29 and acceptor sites at codon nos. 117, 134, and 169, and generates 3 variant transcripts, designated as Exon1A, 1B, and 1C. They encode abbreviated TSPY isoforms harboring in-frame deletions of 87, 104, and 139 amino acids, respectively. The second category of transcripts involves intron skipping events, thereby introns 3 or 4 could be included in the final variant transcripts. They encode variant TSPY proteins harboring a normal amino end but diverge at the intron junctions with short carboxyl termini different from those of most isoforms (Fig. 3C) [16]. To confirm the existence of these variant transcripts and the isoforms detected by Western blot, RT-PCR analysis with transcript-specific primer sets (Table 1) was conducted with total RNAs derived from the same samples, used in the Western blotting (Fig. 3A). Our results showed that these variant transcripts were present in these specimens. Although semiquantitative in nature, the relative amounts of RT-PCR products (Fig. 3B) were similar to those of proteins detected by Western blotting in the respective samples (Fig. 3A). Similar results were obtained for the transcripts for the various germ cell tumor markers, cyclin B1, and controls (Fig. 3B).

3.4. TSPY is an excellent diagnostic marker for TGCTs

The specific expression of TSPY in CIS/ITGCNU and seminoma clearly suggests that this protein can be an effective diagnostic marker for detection and classification of TGCTs. To explore this possibility, we examined TSPY expression in 61 cases of seminomas (42, 9, and 10 cases of stages I, II, and III, respectively) and 70 cases of nonseminomas (embryonal carcinoma, yolk sac tumor, and teratoma at different clinical stages). Of these, 27 cases of seminomas and 26 cases of nonseminomas harbored CIS/ITGCNU adjacent to the respective tumor sites (Table 2). They were independently analyzed from the respective tumor sites within the specimens. Eight cases of nonseminomas contained seminomatous components, which were excluded in the analysis. Immunostaining was performed by a technical staff and independently analyzed by an attending pathologist. The TSPY staining intensity was graded from 0 (none) to 3 (strong); the extent of staining was graded from 0 to 3, representing 0% to more than 60% of tumor cells positive (Table 3). The overall TSPY grading was calculated as the sum of the 2 grades. Hence, this grading system indicates that a score of 0 means no cells were positive for TSPY, whereas a score of 6 means that more than 60% of cells are strongly stained with TSPY antibody. Intermediate scores suggest that staining was either heterogeneous or a variation in intensity on the tumor cells. Our results showed that CIS/ITGCNU showed the most intense and comprehensive staining of the tumor cells (Fig. 4), irrespective of their origins from either seminoma or nonseminoma specimens. In fact, almost every CIS/

Table 1 Primer sets used in RT-PCR analysis of TSPY isoforms, germ cell tumor marker, and reference genes

Primers	Sequence
TSPY-HL2	5′ -GTCTGCGGCGATAGGCCTCCACTT-3′
TSPY-HL3	5′ -TCGGCAGCGGGAAAAGATGGAGCG-3′
TSPY-Exon1A	5′ -GCACAGGCCTTGGTGGAGCTGGAG-3′
TSPY-Exon1B	5′ -GCACAGGCCTTGCGGGAAAAGATGG-3′
TSPY-Exon1C	5′ -GCACAGGCCTTGATGTCAGCCCTG-3′
TSPY-Intron4	5′ -CGGGAAAGGCCTCATCAGGGCTC-3′
PLAP-F	5′ -CAACTTCCAGACCATTGGCTTG-3′
PLAP-R	5′ -TTACCACTCCCACTGACTTCCCTG-3′
KIT-F	5′ -GGACTTGAGGTTTATTCCTGACCC-3′
KIT-R	5′ -GCTTGCTTTGGACACAGACACAAC-3′
OCT4-F	5′ -TGGGGGTTCTATTTGGGAAGG-3′
OCT4-R	5′ -GTTCGCTTTCTCTTTCGGGC-3′
Cyclin B1-F	5′ -TGGGGACATTGGTAACAAAGTCAG-3′
Cyclin B1-R	5′ -TGGGCTTGGAGAGGCAGTATCAAC-3′
HPRT-F	5′ -CCTGCTGGATTACATTAAAGCACTG-3′
HPRT-R	5′ -GTCAAGGGCATATCCAACAACAAAC-3′
CD9-F	5′ -GGATATTCCCACAAGGATGAGGT-3′
CD9-R	5′ -GATGGCTTTCAGCGTTTCCC-3′
FOXM1-F	5′ -TTGCCCGAGCACTTGGAATC-3′
FOXM1-R	5′ -GGGGAGTTCGGTTTTGATGGT-3′
NANOG-F	5′ -TGATTTGTGGGCCTGAAGAAAA-3′
NANOG-R	5′ -GAGGCATCTCAGCAGAAGACA-3′
KRAS-F	5′ -GGACTGGGGAGGGCTTTCT-3′
KRAS-R	5′ -GCCTGTTTTGTGTCTACTGTTCT-3′
WNK1-F	5′ -ACCCTCGGTTGTTCCAGTC-3′
WNK1-R	5′ -ACACATGAGGAGTTGATATGGGA-3′
GAPDH-F	5′ -ATGGGGAAGGTGAAGGTCG-3′
GAPDH-R	5′ -GGGGTCATTGATGGCAACAATA-3′

Table 2 TSPY staining of TGCTs

TGCTs	Stage	Cases	+TSPY	+CIS/ITGCNU
Seminoma	I	42	37	18
	II	9	9	4
	III	10	5	5
Nonseminoma[a]	I	34	4	18
	II	8	0	5
	III	28	6	13

[a] Nonseminoma specimens consisted of embryonal carcinoma, yolk sac tumor, teratoma, and mixed germ cell tumors with seminoma components (which, together with CIS/ITGCNU, were excluded in TSPY immunostaining scoring).

ITGCNU cell was positive for TSPY in most specimens. All but 10 cases of seminomas showed significant and intense staining with the TSPY antibody. The percentages of TSPY negative samples seemed to be higher in the late clinical stage III than in the earlier stages. Nonseminomas, excluding the CIS/ITGCNU or seminoma components present within the specimens, showed only background staining for TSPY. No specific staining pattern could be identified among these minimally stained samples. All samples that were negative for TSPY by immunohistochemistry were also negative in Western blot analysis using the same TSPY antibody. The present results clearly support the notion that TSPY is a significant diagnostic marker for CIS/ITGCNU, the precursor, and seminomas, and a key differential marker for classification of TGCTs.

3.5. TSPY up-regulates chromosome 12p13 genes

Numerous studies suggest that genes on chromosome 12 band p13 could play important functions in the pathogenesis of TGCTs [21-24]. To explore the correlation between the expression levels of TSPY and selected 12p genes, we analyzed available microarray data of normal testis, clinical TGCT, and CIS/ITGCNU samples [26,27] by using hierarchical clustering analysis. Results showed that TSPY expression patterns in clinical and normal samples detected by microarray analyses (Fig. 5A-B, horizontal blue boxes) were closely related to those demonstrated by immunostaining, Western blotting, and RT-PCR studies (ie, Figs. 1-4). TSPY was expressed at moderate levels in normal testes

Table 3 Scoring system for TSPY staining of TGCTs

Staining intensity (SI)	
No staining	0
Weak	1
Intermediate	2
Strong	3
Positive cells (PC)	
None	0
<30%	1
30%-60%	2
>60%	3

Overall score = SI + PC.

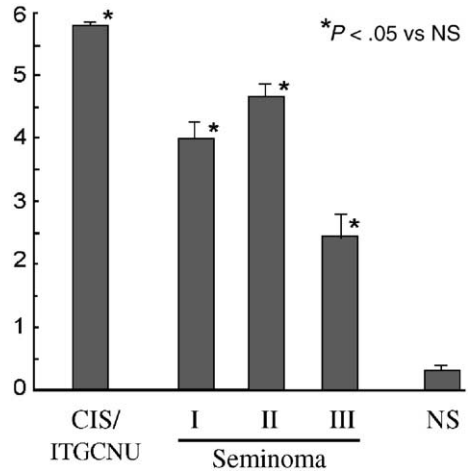

Fig. 4 Relative TSPY immunostaining of tumor cells in various subtypes of TGCTs, based on a crude grading system (as described in Table 3). The CIS/ITGCNU, precursor for all TGCTs, showed the most intense and extensive staining, whereas seminomas at clinical stages I, II, and III were positive but less intense than the CIS/ITGCNU. Nonseminomas at all clinical stages showed minimal staining with the TSPY antibody under the same conditions, suggesting that they did not express TSPY to any significant levels. In early germ cell tumors TSPY seemed to express at higher levels, but declined toward more advanced clinical stages among the CIS/ITGCNU and seminoma samples. Such differential TSPY expression suggests that TSPY could be an efficient marker for identification of premalignant precursors (CIS/ITGCNU) and classification of various subtypes of TGCTs. Student *t* test showed that the *P* value of these samples were less than .05 when compared to nonseminomas (NS), suggesting that they were significantly different from the NS samples in TSPY immunostaining.

(Fig. 5A, NT1-3) and at elevated levels in CIS/ITGCNU (Fig. 5A, ITGCNU1-3), most seminoma (Fig. 5A, SE1 and 5B, SE5-7), and spermatocytic seminoma samples (Fig. 5B, SS1-5), but at reduced levels in most embryonal carcinomas (Fig. 5A, EC1 and 5B, EC2, EC4-6) and 3 seminomas (Fig. 5A-B, SE2-4). Dysgerminomas also showed minimal (background) levels of TSPY hybridization (Fig. 5B, DG1-3). Because dysgerminomas are female ovarian seminomatous germ cell tumors lacking the Y chromosome, it is expected that no TSPY should be expressed in them. It is interesting to note that some testicular seminomas, such as SE2-3 (Fig. 5A) and SE4 (Fig. 5B) showed reduced levels, whereas some nonseminomas, such as EC3 (Fig. 5B), showed elevated levels of TSPY expression. Such heterogeneity in TSPY expression was also observed in immunostaining studies (Table 2 and Fig. 4). Hence, TSPY expression patterns detected by microarray analyses of clinical samples are similar to those observed via immunologic techniques.

Previously, we demonstrated that ectopic expression of TSPY in cultured HeLa cells up-regulated various oncogenes and progrowth genes, including CCND2, and repressed apoptotic genes and growth inhibitors [25]. A

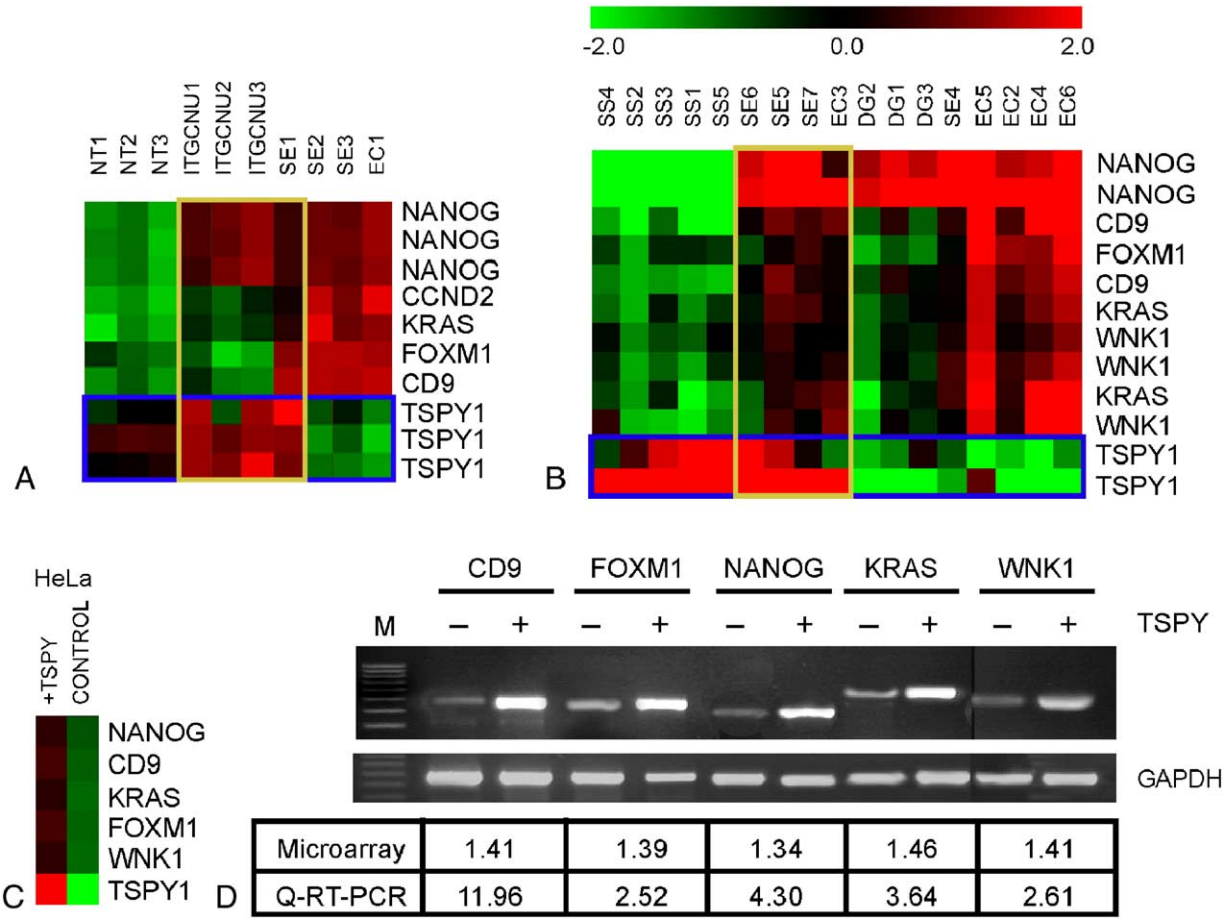

Fig. 5 Expression of TSPY is associated with up-regulation of selected chromosome 12p genes, involved in TGCTs. (A-B) Hierarchical clustering analysis of microarray data on TSPY and selected 12p13 genes from GEO data sets (A, see Materials and methods) and published data (B, Looijenga et al [27]). Gene candidates among the list may occur more than once because of duplicate probes, indicating the consistent trend of gene expression in the group. The expression patterns of CIS/ITGCNU and seminoma samples (vertical boxed regions in A and B) closely resemble that for HeLa cells ectopically expressing TSPY transgene (C). Ectopic TSPY expression up-regulates 12p genes. (C) Hierarchical clustering analysis of selected 12p genes in HeLa cells overexpressing TSPY (HeLa) and control cells (control). (D) Semiquantitative RT-PCR analysis of RNAs derived from HeLa cells expressing (+) and lacking (−) TSPY, using specific primers for 12p genes. GAPDH was used as a control. The relative ratios of TSPY expression were calculated as signals from HeLa + TSPY/HeLa + control, as from microarray and Q-RT-PCR analyses (lower panel).

reexamination of our microarray data showed that selected 12p genes—including the CCND2 (cyclin D2), KRAS (v-Ki-ras2 oncogene), NANOG (a homeobox stem cell factor), FOXM1 (the forkhead box M1), WNK1 (WNK lysine deficient protein kinase 1), and CD9 (a member of the transmembrane 4 superfamily)—were also up-regulated either directly or indirectly in HeLa cells by the ectopic expression of TSPY (Fig. 5C). To confirm such up-regulation, specific primer pairs corresponding to the respective transcripts of these genes (Table 1) were used in a semiquantitative RT-PCR analysis of RNAs derived from HeLa cells harboring/expressing TSPY (Fig. 5D, +) and those containing the vector alone (Fig. 5D, −). Our results confirmed the initial microarray analysis of these cell populations (Fig. 5D) and showed higher levels of transcripts of these 12p genes in HeLa cells expressing TSPY than cells harboring the vector alone. Interestingly, the expression levels of these genes, estimated by semiquantitative RT-PCR, were higher than those estimated by our initial microarray analysis, as previously observed [25]. We surmise that these differences could be attributed to the respective sensitivity of the 2 methods.

Expression of the selected 12p genes was low in normal testes (Fig. 5A, NT1-3), increased in CIS/ITGCNU (Fig. 5A, ITGCNU1-3) and further in seminomas (Fig. 5A-B, SE1, SE4-7), and was at highest levels in the nonseminomatous embryonal carcinomas (Fig. 5A-B, EC1-6). The gradual increases in the expression of 12p genes could be associated with the progression of the tumorigenic process(es) of TGCTs. Significantly, the expression patterns of TSPY and selected 12p genes in CIS/ITGCNU and most seminoma samples (Fig. 5A-B, vertical yellow boxes) resembled closely that of the HeLa cells ectopically expressing TSPY (Fig. 5C), suggesting a

possible link between elevated TSPY expression and up-regulation of the selected 12p genes in these types of germ cell tumors. Notably, further increases in 12p gene expression in some seminomas (ie, SE2-4) and most embryonal carcinomas were associated with reduced TSPY expression in these samples. Because gain of chromosome 12p and/or amplification of certain portion of this chromosome were associated with the development of advanced or invasive germ cell tumors, such differential expression of TSPY suggests that it might play a significant, but temporal, role in the evolution of the tumorigenic germ cell genome.

4. Discussion

The present study demonstrated the significant association of TSPY expression with TGCT precursor, CIS/ITGCNU, and most testicular seminomas. Examination of available expression microarray data confirmed such preferential expression pattern of this Y chromosome gene in different types of TGCTs. TSPY is coexpressed with other established germ cell tumor markers (ie, PLAP, c-KIT, OCT4, and the proliferative marker, Ki-67) in most tumor cells [19,32,34], thereby confirming its significance as a diagnostic marker for CIS/ITGCNU and seminomatous tumors. By establishing a crude grading system, we were able to efficiently demonstrate such differential TSPY expression and to distinguish seminomas from nonseminomas. Hence, TSPY is an important diagnostic marker for the detection and/or classification of various subtypes of TGCTs for routine pathologic and clinical analysis.

Gonadoblastoma is a rare germ cell tumor occurring most frequently in XY sex-reversed and intersex patients who harbor residual Y chromosome materials [4-6,8-10]. Gonadoblastoma and TGCT precursor, CIS/ITGCNU, share significant similarities in their morphology and tumor behavior [24,35,36]. The abundant expression of TSPY in both gonadoblastoma and CIS/ITGCNU further supports such postulation of common origin(s) of these 2 precursors for aggressive germ cell tumors [15,36]. The identification of TSPY as a significant candidate for GBY thereby associates this repeated Y chromosome gene with the pathogenesis of the more common TGCTs among young men. Because CIS/ITGCNU has been considered as the precursor for both seminomas and nonseminomas (ie, embryonal carcinoma, teratoma, and yolk sac tumor [15,37-39]), the differential expression of TSPY between seminomas and nonseminomas implies that these 2 types of germ cell tumors have taken on separate differentiation pathways in their tumorigenic processes. These findings suggest that testicular seminomas evolve along the germ cell lineage, whereas nonseminomas could be activated to more pluripotent states capable of addition differentiation into other cell types [19,24]. Potentially, these adult pluripotent cells may be used as somatic stem cells for transdifferentiation studies, including those for gonocytes [17].

Several studies have demonstrated the expression of TSPY in gonadoblastomas, testicular seminomas, intracranial germ cell tumors, prostate cancer, hepatocellular carcinoma, and melanoma of male origins [13,14,16,40-42]. Previously, we demonstrated a preferential expression of a variety of alternatively spliced TSPY transcripts coding for different abbreviated isoforms of TSPY protein in prostate cancer samples [16]. Interestingly, similar enrichment of TSPY isoforms at both RNA and protein levels in seminoma samples were also observed in the present study, suggesting the same preferential expression of the abbreviated forms of this Y-encoded protein in this type of TGCTs. Currently, it is uncertain if these polymorphic TSPY proteins serve the same or different biologic function(s). It will be interesting to determine if tumors in other tissues (eg, intracranial, hepatocellular carcinoma, and melanoma) also preferentially express such abbreviated forms of the TSPY protein.

TSPY is expressed in early gonocytes in prenatal and postnatal testes [32] and spermatogonia and, to a certain extent, round spermatids of adult testis [28]. It has been postulated to serve a certain role(s) in stem germ cell proliferation and/or male meiosis [4,7]. In particular, disruption/delay of fetal germ cell development could play a significant role in the pathogenesis of TGCTs [37,43-45]. Hence, TSPY expression in normal fetal gonocytes/prespermatogonia and adult spermatogonial stem cells affirms its possible functions in male stem germ cell differentiation, whereas its ectopic expression in the TGCT precursor, CIS/ITGCNU, and seminomas supports the possibility of a delayed or a reactivated fetal programming in these tumor germ cells. Currently, the exact mechanisms of TSPY action(s) at the molecular and cellular levels are uncertain; its expression in germ cell tumors and cancers of somatic origins suggests that it might exert a proliferative function(s) at the cellular level when it is ectopically expressed in these cells. Indeed, we had recently shown that overexpression of TSPY in cultured somatic cells potentiated cell proliferation in vitro and tumor formation in nude mice [25]. Cells overexpressing TSPY transited the G_2/M phase more rapidly than those without such expression. Transcriptome analysis demonstrated that progrowth genes and selected oncogenes were up-regulated, whereas apoptotic factors and cell cycle inhibitors were down-regulated in these TSPY expressing cells. Ontology analysis of the differentially expressed genes suggested that pathways involved in cell cycle regulation were mostly affected.

A more focused analysis in the present study demonstrated that ectopic expression of TSPY in HeLa cells up-regulated selected chromosome 12p genes, postulated to be involved in the evolution of TGCTs [21,24]. Currently, the exact mechanism(s) by which TSPY alters the expression these chromosome 12p genes is unknown. TSPY has not been demonstrated as a transcription factor, and hence, it

could likely influence such changes in gene expression in an indirect manner(s). Furthermore, it is interesting to observe that GAPDH is also located at chromosome 12p13, and it was not up-regulated in HeLa cells overexpressing the TSPY transgene (Fig. 5D). Hence, TSPY effects could selectively occur on genes residing in this chromosome region. Significantly, among these 12p genes up-regulated by ectopic expression of TSPY, both CCND2 and FOXM1 are key players in cell cycle regulation. CCND2 is the gene for cyclin D2 that binds to and activates the cyclin-dependent kinase, CDK4/6, essential for the cell to exit G_0 and enter G_1 or to cycle from G_1 to S phase [46,47]. By up-regulating cyclin D2, TSPY could have a positive effect(s) on cell proliferation and tumor initiation. Significantly, FOXM1 encodes a key transcription factor directly binding to the promoters and regulating the transcription of many genes coding for various cell cycle regulators (including cyclin B1, Polo-like kinase 1, CENP-F, Cdc25B phosphatase, and Auro B kinase) important for G_2/M stage [48-50]. The up-regulation of FOXM1 by ectopic TSPY expression might be responsible for the rapid transition of the cells through G_2/M, previously demonstrated [25]. More importantly, an expedited progression through this stage of the cell cycle could affect various G_2 and mitotic checkpoints, essential for DNA repair and orderly cell division [51,52]. Inactivation of such checkpoints will likely enhance chromosome nondisjunction and/or genomic instability, thereby increasing mutational events and exacerbating the tumorigenic process(es) [53]. We surmise that such TSPY effects could be important for the premalignant precursor (ie, CIS/ITGCNU) to enter the cell cycle and for seminomatous cells to maintain their oncogenic properties. The mutational pressure exerted by ectopic TSPY expression could result in either a gain of chromosome 12p or amplification of certain genes therein that favors tumorigenic progression. Indeed, gain/amplification of chromosome 12p seems to be related to acquisition of pluripotency or "stemness" properties by the tumor germ cells [20,54,55]. The development of aggressive/pluripotent phenotypes could thereby minimize the necessity for TSPY functions, resulting in reduction in its expression in late stages of TGCTs.

Currently, we are uncertain what role TSPY might play in spermatocytic seminoma. This type of tumor is postulated to derive from primary spermatocytes, and gain of chromosome 9 is the only consistent genomic anomaly [27]. In fact, 12p genes are not up-regulated in this type of germ cell tumors (Fig. 5B). TSPY could potentially affect other genes residing elsewhere in the human genome that favor the oncogenic process(es) for this type of seminomas. Nevertheless, TSPY dysregulation of the cell cycle, in combination with other oncogenic events, including activation of proliferation and stem cell genes as well as expression of specific cell cycle controlling micro-RNAs [56], could play critical roles in the pathogenesis and/or progression of gonadoblastoma and TGCTs.

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Reprint 8

THE TSPY GENE FAMILY

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Abstract

The testis-specific protein Y-encoded (*TSPY*) gene is one of the early genes identified on the human Y chromosome. It is tandemly repeated on the short arm of this chromosome, postulated to contain the gonadoblastoma locus responsible for predisposing dysfunctional germ cells to tumorigenesis. *TSPY* encodes a phosphoprotein harboring a conserved domain, termed SET/NAP, present in various proteins involved in cell cycle regulation, chromatin modeling, and transcription regulation. Six TSPY-like genes have been identified in different mammalian genomes. One in particular, designated as *TSPX*, is located on the syntenic region of the X chromosome. Both *TSPY* and *TSPX* maintain similar gene organization with 6 and 7 exons respectively. *TSPX* encodes a protein with two additional domains, a N-terminal proline-rich domain and a carboxyl bipartite acidic domain, that are absent in TSPY. Both *TSPY* and *TSPX* possess contrasting properties in cell cycle regulation when they are ectopically expressed. Other autosomal members of this gene family are single-exon genes, postulated to be retrotransposons of *TSPY*. They encodes similar-sized proteins that share high levels of homology at their SET/NAP domains, but diverge at the flanking regions. Specific mutations on the *TSPY*-like 1 gene have been demonstrated to be responsible for the sudden infant death with dysgenesis of the testes syndrome. Hence, *TSPY* and *TSPY*-like genes are hypothesized to serve a variety of different physiological functions mediated by the conserved SET/NAP and unique domains in their respective proteins.

The Gonadoblastoma Locus and the TSPY Gene on the Human Y Chromosome

Gonadoblastomas are benign tumors consisting of aggregates of large, round germ cells and immature Sertoli/granulosa cells surrounded by ovarian-type stroma (Scully, 1953; Scully, 1970) that arise most frequently (up to ~66%) in the dysgenetic gonads of intersex individuals who harbor in their genome either the entire or a partial Y chromosome (e.g., 46,XY or 45,X/46,XY) (Verp and Simpson, 1987). Patients with Turner syndrome who have a 45,XO chromosome complement and residual Y chromosome material are also at risk for developing gonadoblastoma, although at a much lower rate (7–10%) (Gravholt et al., 2000; Mazzanti et al., 2005). These tumors are capable of synthesizing sex hormones, such as estrogen or testosterone, leading to either feminization or virilization of the dysgenetic gonads, respectively (Verp and Simpson,

1987; Mancilla et al., 2003). Approximately 80% of intersex patients are phenotypic females and 20% are phenotypic males (Verp and Simpson, 1987).

In 1987, David Page proposed the existence of the gonadoblastoma locus on the Y chromosome (GBY) to explain the high frequency of gonadoblastoma in the dysgenetic gonads of XY females (Page, 1987). The *GBY* gene is postulated to serve a normal function in the testis, but could predispose the dysgenetic gonads of intersex individuals to tumorigenesis (Page, 1987; Lau, 1999) . Gonadal dysgenesis is thought to arise from mutation or deletion of the sex determining region Y (*SRY*) gene (Sinclair et al., 1990) or other downstream sex determining genes while the *GBY* gene remains in the XY or XO/XY mosaic genome. Because normal females do not have the Y chromosome, the acquisition of the *GBY* gene could be considered as a gain of function in the dysgenetic gonads in female. For males, the *SRY* gene could be functional, while other sex determining genes downstream of this primary switch gene might be affected, resulting in streaked or dysgenetic gonads/testes. Based on these hypotheses, the *GBY* gene potentially could act as an oncogene or tumor-promoting gene in dysfunctional germ cells of sex-reversed or testis dysgenesis individuals. Deletion mapping narrowed the location of the GBY locus to a small region on the short arm and proximal region on the long arm of the Y chromosome (Salo et al., 1995; Tsuchiya et al., 1995; Lau, 1999). The transcriptional units of the testis-specific protein Y-encoded (*TSPY*) repeated gene (Arnemann et al., 1991; Zhang et al., 1992) are primarily located on this GBY critical region, thereby positioning *TSPY* to be a significant candidate for this oncogenic locus. Indeed, *TSPY* is abundantly expressed in gonadoblastoma, testicular seminoma, and exxtragonadal germ cell tumors of male origin (Schnieders et al., 1996; Hildenbrand et al., 1999; Lau et al., 2000; Honecker et al., 2005; Kersemaekers et al., 2005; Hoei-Hansen et al., 2006) and possesses proliferative properties that could function as an oncogene/tumor promoter in human oncogenesis (Oram et al., 2006). Hence, studies of a relatively rare and special form of germ cell tumor, gonadoblastoma, have identified a significant candidate gene that potentially could play an important oncogenic role(s) in the more common testicular germ cell tumors (TGCTs).

Properties of the Testis-Specific Protein Y-Encoded (TSPY) Gene

Early studies of the *TSPY* gene demonstrated that its functional units are 2.8-kb in size and consist of 6 exons and 5 introns (Zhang et al., 1992; Schnieders et al., 1996) embedded in repeat units of ~20.5-kb EcoR1 DNA fragments (Zhang et al., 1992). Sequencing of the male-specific region of the Y chromosome (MSY) in humans showed that *TSPY* structural gene is embedded in a 20.4-kb unit that is tandemly repeated ~35 times on the GBY critical region of a reference individual (Skaletsky et al., 2003). Although the number of repeats varies among individuals and could range from 23 to 49 (Repping et al., 2006), these repeat units show >98% sequence homology among both among *TSPY* transcriptional units and their flanking regions. They constitute the largest and most homologous protein-coding tandem array, so far identified in the human

genome and represent close to half of the 78 transcriptional units identified within the MSY on the Y chromosome (Skaletsky et al., 2003).

TSPY has been postulated to serve a normal function in directing the spermatogonial cells to enter meiosis (Schnieders et al., 1996; Lau, 1999). Recent studies suggested that TSPY could possess additional mitotic function in the proliferation of embryonic gonocytes and adult spermatogonia (Honecker et al., 2004). *TSPY* gene is evolutionary conserved on the Y chromosome of all placental mammals, such as the primates and artiodactyls (Vogel et al., 1997), except the rodents. Similar to the human situation, they are repetitive genes on the Y chromosome. The rat has one functional *Tspy* gene on its Y chromosome (Dechend et al., 1998; Mazeyrat and Mitchell, 1998). In the murines, several *Apodemus* species and *Mus platythrix* posses a functional Tspy gene while other species in the *Mus* subgenus, including the laboratory mouse (*Mus musculus*), harbor an apparently nonfunctional (with in-frame and splice junction mutations) *Tspy* gene on their Y chromosomes (Schubert et al., 2000). Several autosomal *TSPY*-like (*TSPY-L*) genes have been identified in both the mouse and human genomes (Vogel et al., 1998b). Currently, it is uncertain which of these *Tspy*-like genes could serve as a functional counterpart for *Tspy* in the mouse.

TSPY encodes a predominant 38-kDa phosphoprotein of 308 amino acids. It shares tight homology to the product of the *SET* oncogene, initially identified from an intrachromosome 9 translocation in a patient with undifferentiated leukemia (von Lindern et al., 1992; Adachi et al., 1994a; Adachi et al., 1994b). The SET oncoprotein is a 39-kDa phosphoprotein consisting of 277 amino acids. It has also been identified as the template activating factor Iβ (*TAF-Iβ*), a host protein required for DNA replication and transcription of the adenovirus genome (Nagata et al., 1995). Together with the nucleosome assembly protein-1 (NAP-1), they are founding members of a protein family designated as TSPY/SET/NAP (Vogel et al., 1998b; Lau, 1999; Ozbun et al., 2001). Members of this protein family harbor a conserved domain of ~160 amino acids, termed SET/NAP domain that binds to the type B cyclins and core histones (Kellogg et al., 1995; Matsumoto et al., 1999). They serve a diverse spectrum of functions, including DNA replication, transcription modulation/chromatin modeling, and cell cycle regulation (Nagata et al., 1995; Compagnone et al., 2000; Chai et al., 2001; Zhang et al., 2001; Canela et al., 2003;Oram et al., 2006). Mutations and/or dysregulation of members of this SET/NAP gene family have been associated with various forms of human cancers, i.e. leukemia for *SET* oncoprotein and cell division autoantigen-1 (*CDA1)* gene (von Lindern et al., 1992; Adachi et al., 1994a; Chai et al., 2001), lung cancer for the differentially expressed nucleolar TGF-β1 target (*DENTT*) gene (Ozbun et al., 2003), and gonadoblastoma, testicular germ cell tumors, prostate cancer (Lau et al., 2003), melanoma (Gallagher et al., 2005) and hepatocellular carcinoma (Yin et al., 2005) for the *TSPY* gene.

TSPX is An X-Located *TSPY* Homologue with Contrasting Properties

Four laboratories have independently isolated and characterized an X-located gene (at Xp11.22), recently designated as *TSPX* (Delbridge et al., 2004), that shares significant homologies with *TSPY* and *SET*. In all instances, *TSPX* was isolated by exploratory cloning strategies and was designated separately as the cutaneous T-cell lymphoma-associated tumor antigen, *SE20*-4, (Eichmuller et al., 2001), *CDA1* gene (Chai et al., 2001) and *DENTT* gene (Ozbun et al., 2001) respectively. Both *CDA1* and *DENTT* have been studied in significant detail. Although they were described as possessing a SET/NAP/TSPY domain in their encoded proteins, their identity as the homologue of *TSPY* was not obvious until the structure and mapping of these genes were studied recently (Delbridge et al., 2004).

TSPX has been mapped to the syntenic region harboring the homologues of other Y-genes on X chromosome in humans and mice. Both *TSPX* and *TSPY* maintain similar gene organization (Figure 1). The *TSPX* gene is about 6.3-kb in size and harbors 7 exons. It encodes an ~80 kDa protein of 693 amino acids. *TSPY* is 2.8-kb in size and harbors 6 exons. It encodes a variety of polymorphic proteins with the main product being a protein with 308 amino acids and a calculated molecular weight of 35-kDa. The homologous regions of both TSPX and TSPY are encoded by exons 2-5 of both genes and have highly conserved sequence homology and exon-intron organization. These portions of the proteins also harbor the SET/NAP domain. TSPX differs from TSPY by having an N-terminal proline-rich domain and a carboxyl bipartite acidic domain, encoded by exon 1 and exons 6-7 respectively. Both proteins are phosphorylated, presumably by cyclin dependent kinases (CDKs) (Chai et al., 2001) and other protein kinases (Krick et al., 2006). TSPY could be located in both cytoplasm and nuclei of expressing cells. The phosphorylation of the tyrosine at residue #300 of the predominant TSPY isoform by CK2 kinase has been demonstrated to be essential for its nuclear translocation (Krick et al., 2006).

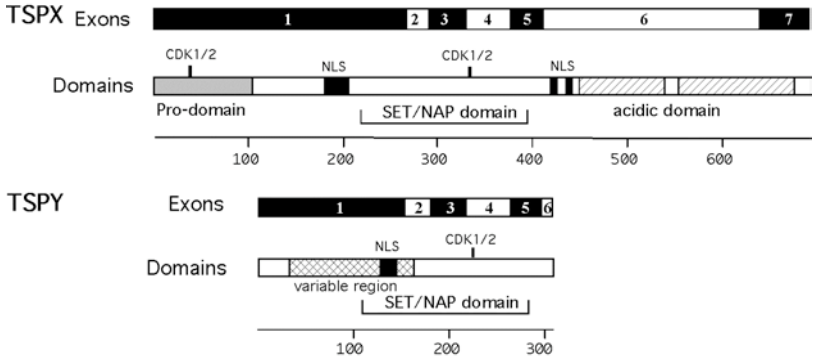

Figure 1. Organization of human *TSPX* and *TSPY* exons and corresponding protein domains.

Significantly, over-expression of *TSPX* (*CDA1*) arrests cell growth at G_2/M phase of the cell cycle (Chai et al., 2001). TSPX is localized at the nucleus and nucleolus of the cells. Such cell growth inhibitory effects were mapped to the carboxyl acidic domain and two CDK-1/2 phosphorylation sites, located at the proline-rich domain and SET/NAP domain respectively. The latter phosphorylation site is also conserved at the SET/NAP domain of the TSPY protein. Targeted mutations of both phosphorylation sites or truncation of the acidic domain of TSPX eliminates its growth inhibition. Importantly, over-expression of the SET protein also arrested cell proliferation, specifically at G_2/M phase (Canela et al., 2003). Such growth inhibitory effects of SET were also mapped to its acidic carboxyl domain (Canela et al., 2003), thereby ascertaining the importance of the acidic carboxyl domain of both SET and TSPX in cell cycle regulation. SET binds the mitotic cyclin B and, in collaboration with other factors, affects the cyclin B-CDK1 activities (Canela et al., 2003; Carujo et al., 2006). Normally, *TSPX* (*DENTT*) is expressed in a wide spectrum of tissues, with major sites in brain, lung, thymus, adrenal, pituitary, smooth muscle, testis and ovary in adult mice. It is initially expressed in the heart and the primitive brain of E8 mouse embryos. The embryonic expression expands gradually to other tissues at later stages and reaches the ubiquitous pattern in adults (Ozbun et al., 2003; Ozbun et al., 2005).

Over-expression of *TSPY* potentiates cell proliferation in cultured cells and promotes tumor growth in nude mice (Oram et al., 2006). It probably mediates such proliferative effect(s) at the G_2/M checkpoints since cells over-expressing *TSPY* transit G_2/M phase more rapidly than those repressing its expression. These cell cycle effects are in contrast with those of *TSPX* (*CDA1*). Both genes encode relatively homologous proteins with a conserved SET/NAP domain, with the exception of the carboxyl acidic tail that is absent in TSPY. Since the cell cycle inhibitory effect(s) of TSPX has been mapped to this portion of the molecule, the differences in cell cycle properties between TSPX and TSPY could be attributed to the presence and absence of the carboxyl acidic domain in the respective proteins.

Microarray analysis demonstrates that ectopic TSPY expression primarily affects genes in three cellular processes, cell cycle regulation, phosphate transport and neuromuscular development (Oram et al., 2006). Among the cell cycle genes, *TSPY* up-regulates several oncogenes (epidermal growth factor receptor (*ERBB*), and members of the *WNT* and *RAS* oncogenes), growth factors (*PDGFC*, *EGF*-related, *ANKRD15*, *RGC32*, *NANOS1*) cyclin D2, and a histone acetyltransferase (*EP300*), an apoptosis inhibitor (*GSPT1*) and an antigen (*CD24*) highly expressed in small cell lung carcinoma. The down-regulated genes include an inhibitor for *CDK4*/*CDK6*, transforming growth factor β3, a pro-apoptotic factor (*IGFB3*), and an inhibitor of MAP kinases (dual specificity phosphatase 5). In particular, the *CCND2* gene (that encodes cyclin D2) and another up-regulated gene, the tetratricopeptide repeats (*TMTC1*), reside on chromosome 12p, which is frequently amplified and expressed at high levels in testicular germ cell

tumors. Cyclin D2 complexes with CDK4 or CDK6 to mediate G_1/S transition and promote cell proliferation. *CCND2* was up-regulated in cells over-expressing *TSPY*. Conversely, *CDKN2B* encodes an inhibitor (INK4B) of cyclin D2 activities by acting on CDK4 and CDK6. It is down-regulated in the same cells. Loss of INK4 kinase inhibitors has been postulated to be important for progression from carcinoma-in-situ (CIS) to invasive germ cell tumors (Bartkova et al., 2000). Significantly, *NANOS1* is the homologue of the *Drosophila nanos* gene that is critical for development and maintenance of germ stem cells in both sexes. It is expressed in the spermatogonia and spermatocytes of human testis, and has been postulated to be important for development of germ stem cells in humans (Jaruzelska et al., 2003). The up-regulation of this gene is extremely interesting as it supports the role of *TSPY* in germ stem cell biology.

Studies so far suggest that *TSPX* is expressed in normal and cancerous somatic cells (Chai et al., 2001; Eichmuller et al., 2001; Ozbun et al., 2001; Ozbun et al., 2003; Ozbun et al., 2005) while *TSPY* is expressed primarily in germ cells (Zhang et al., 1992; Schnieders et al., 1996; Lau et al., 2000; Honecker et al., 2004) and tumors of male origin (Lau et al., 2003; Gallagher et al., 2005; Yin et al., 2005; Hoei-Hansen et al., 2006). To determine if they could be co-expressed in testicular germ cell tumors and their precursor CIS, we examined the expression patterns of TSPY and TSPX by immunofluorescence analysis of 10 cases of seminoma and 4 cases of nonseminomas consisting of yolk sac tumors and embryonal carcinomas, some of which also harbored adjacent CIS. TSPX was not expressed in any of these germ cell tumors, except in one case where there was significant infiltration of lymphocytes into the germ cell

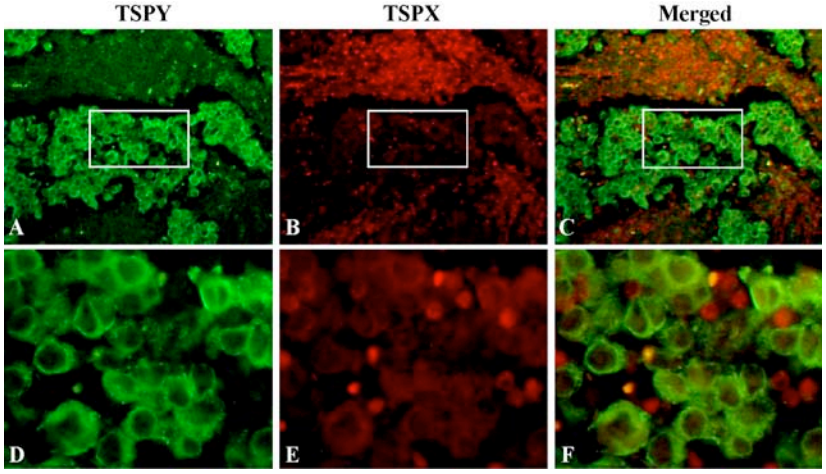

Figure 2. TSPY and TSPX are differentially expressed in a case of human seminoma. Immunostaining shows that TSPY and TSPX are rarely expressed together in testicular germ cell tumors. In a rare case of seminoma with lymphocyte infiltration of the germ cell tumor in which TSPY (**A** and **D**, green) and TSPX (DENTT) (**B** and **E**, red) expression were detectable by immunofluorescence, TSPY was primarily located on the tumor germ cells while TSPX was detected mainly on the nuclei of the infiltrating lymphocytes. They

did not show any significant co-localization of their respective proteins in the same cells. TSPY monoclonal antibody #7 (Kido and Lau, 2005) and DENTT (TSPX) polyclonal antibody (AbCam, ab10318) were used for immunofluorescence analysis. **C** is a merged image of **A** and **B**. Boxed areas in **A**, **B** and **C** are enlarged in **D**, **E**, and **F** respectively.

tumor (Figure 2). TSPX was expressed in the nuclei of the lymphocytes while TSPY was positive in the tumor germ cells. Such mutual exclusion raises the possibility that the cell cycle functions of TSPY and TSPX could be antagonistic in testicular germ cell tumors.

Organization of TSPY-Like Genes and Proteins

Currently six *TSPY*-like genes have been identified in the genomes of humans and several species of mammals (Table 1). *TSPY*-Like 2 (*TSPY-L2*) is *TSPX* and is evolutionarily conserved on the X chromosome of all mammals, except cattle, which harbors a highly homologous *TSPY-L2* gene on chromosome 25. All other *TSPY*-like genes are located on various autosomes, except for *TSPY-L6* that is absent in the rodents. These genes encode proteins of slightly different sizes that harbor a SET/NAP domain and belong to a cluster of orthologous group (COG) of proteins, termed DNA replication factor/protein phosphatase inhibitor SET/SPR-2 (KOG1508) (Tatusov et al., 1997; Tatusov et al., 2003), the members of which are present in many eukaryotes, including mammals, worms, puffer fish and fruit flies. The *TSPY* and *TSPY*-like genes constitute a subfamily of this COG. Only *TSPY* and *TSPX* harbor both exons and introns in their respective genes (Figure 3A). The primordial *TSPY-L2* gene could have derived from a common ancestral gene with SET/TAF-Iβ and NAP-1 and resided on a pair of chromosomes that evolved into the proto-X and proto-Y chromosomes (Figure 4). During the evolution of the sex chromosomes, *TSPY* might have lost the N-terminal

Table 1. Chromosomal location of *TSPY/TSPY-like* and *SET* genes among various mammals.

	Human (46,XY)	Chimpanzee (48,XY)	Mouse (40,XY)	Rat (42,XY)	Dog (78,XY)	Cattle (60,XY)
TSPY-L1	6	5	10	20	12	5
TSPX/TSPY-L2	X	X	X	X	X	25*
TSPY-L3	20	21	2	3	24	13
TSPY-L4	6	5	10	20	12	Scaffold^{+}
TSPY-L5	8	7	15	7	29	12
TSPY-L6	2	12	-	-	10	11
TSPY	Y	Y	Y	Y	Y	Y
SET/TAF-Iβ	9	11	2	3	9	11

TSPY-like genes are identified by sequence homology analysis with respective human/mouse genes. Chromosome mapping was performed with BLAT analysis of available genome sequence databases of respective mammals at http://www.genome.ucsc.edu/ (Kent, 2002; Hinrichs et al., 2006). $^{+}$Scaffold, sequence identified but not mapped on to any chromosome. *Provisional.

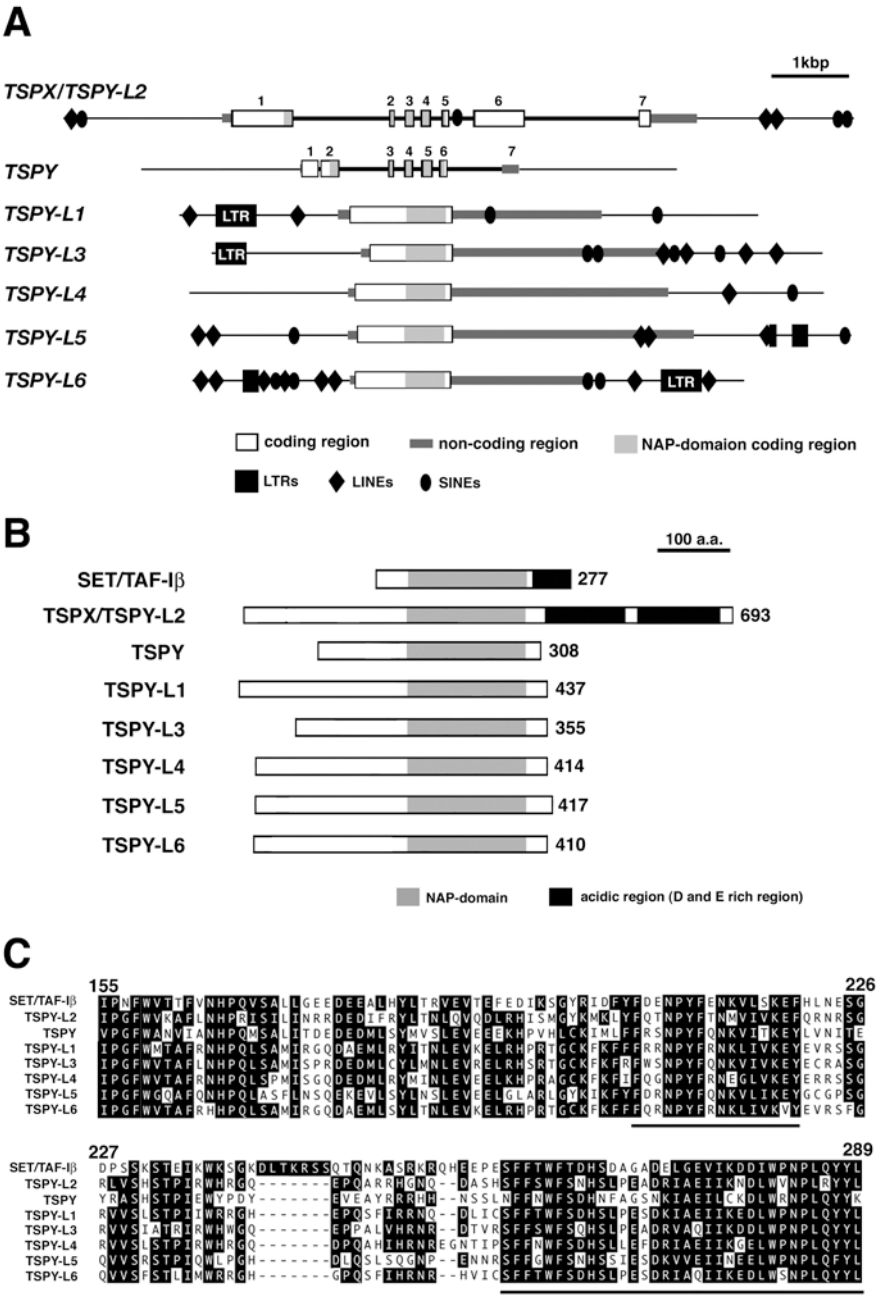

Figure 3. Gene organization, protein structure and homologous domains of human *TSPY*, *TSPY*-like and *SET/TAF*-Iβ genes. **A**. *TSPY* and *TSPX/TSPY-L2* are the only two genes of this family containing exons interrupted by introns. *TSPY-L1* and *TSPY-L3-6* are single-exon genes, arose from possibly retrotransposition of *TSPY* transcripts. Numerous LTRs, LINEs and SINEs, characteristics of

retrotransposons, are present in their flanking sequences (identified by the web-based program, RepeatMasker developed by AFA Smit, R Hubley & P Green, http://repeatmasker.org). **B**. Protein structures of TSPY family. All members of the TSPY gene family encode protein products that are highly homologous at their carboxyl portions harboring a SET/NAP domain. The N-terminal portions are unique to the individual members. TSPX/TSPY-L2, similar to SET/TAFIβ, contains an additional acidic domain at its carboxyl terminus. The number of amino acids for each protein is listed immediately after the carboxyl end of the respective protein. **C**. Protein alignment of the conserved SET/NAP domain of the human TSPY family members. The numbers at the top correspond to the residue positions of the human TSPY protein. Two highly conserved regions are underlined. The accession numbers for the protein sequences are: TSPY, NP_003299; TSPY-L1, NM_003309; TSPX/TSPY-L2, AF254794; TSPY-L3, BC101556; TSPY-L4, NM_021648; TSPY-L5, NM_033512; TSPY-L6, BC068576; SET/TAF-Iβ, M93651.

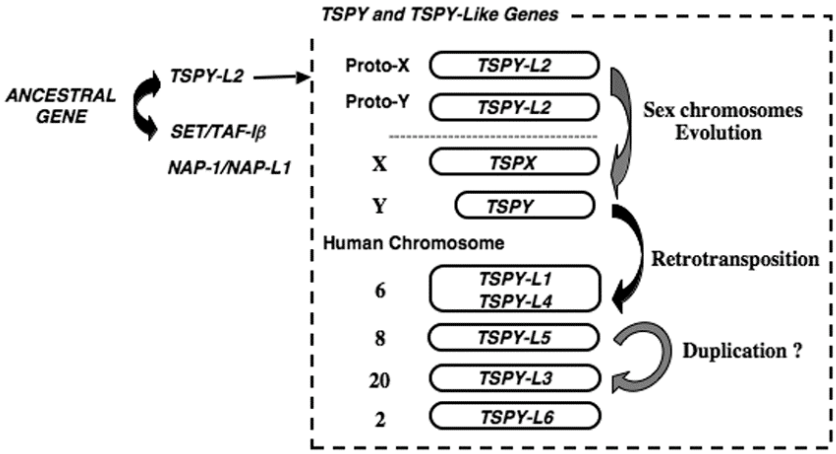

Figure 4. Diagrammatic illustration of probable evolutionary events for the TSPY and TSPY-like genes. The primordial TSPY-L2 gene probably shared common ancestral gene as the SET/TAF-I and NAP and related genes. It was located on a pair of proto-X and proto-Y chromosomes that eventually evolved into the present day X and Y chromosome. TSPX had maintained the structure of the primordial TSPY-L2 gene while TSPY had lost its carboxyl acidic domain before it was amplified on the Y chromosome. The autosomal TSPY like genes arose from retrotransposition of TSPY transcripts since they encode proteins with a conserved SET/NAP domain, but lack a carboxyl acidic domain, present in TSPX.

proline-rich and C-terminal acidic domains before its sequence was amplified on the Y chromosome while the *TSPX/TSPY-L2* gene had maintained structural similarity to the common ancestral gene into the present. The autosomal TSPY-like genes have a single-exon and could have derived from *TSPY* via retrotransposition events as their encoded proteins lack the carboxyl acidic domain present in TSPX (Figure 3B). Indeed numerous long terminal repeats (LTR) retrotransposons, and non-LTR long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs) transposon sequences (Kazazian, 2004; Ohshima and Okada, 2005) are scattered around the flanking regions of the respective TSPY-like genes (Figure 3A), supporting the postulation that they were

derived from retrotransposition of transcripts originating from this Y chromosome gene. Alternatively, these *TSPY*-like genes could have derived from duplication of the initial retrotransposon, resulting in multiple single-exon genes on the autosomes. Except for *TSPX/TSPY-L2*, all *TSPY*-like genes encode proteins that are highly conserved at their carboxyl termini, but variable at their N-termini (Figure 3B). Alignment of their protein sequences suggests that homologies are present only at the 160-residue SET/NAP domain and the short flanking regions (Figure 3C). The conservation of this domain among the TSPY and TSPY-likes proteins suggests that it must have an important role(s) in the biological functions of this protein subfamily. Specific mutations of the *TSPY-L1* gene on chromosome 6 have been identified with the sudden infant death with dysgenesis of the testes (SIDDT) syndrome (Puffenberger et al., 2004). Notably, one frameshift mutation truncates the coding sequence for the SET/NAP domain from its open reading frame. The mutated protein is incapable of translocation to the nuclei and might contribute to the disease process. Further studies demonstrate that TSPY-L1 interacts with the ZFP106 at its SET/NAP domain and N-terminal flanking region, important for such nuclear translocation and formation of nuclear bodies of undefined function(s) (Grasberger and Bell, 2005). Currently the exact functions of *TSPY* and *TSPY*-like genes are uncertain. As discussed above, TSPY and TSPX possess contrasting functions in cell cycle regulation that have been attributed to the absence and presence of an acidic domain at their respective carboxyl termini. Hence, the unique portions, flanking the conserved SET/NAP domain, of the TSPY and TSPY-like proteins could be responsible for specifying the distinct functions of the respective members of the protein family.

Expression of *TSPY* and *TSPY*-Like Genes in the Mouse

To explore the possible functions of *TSPY* and *TSPY*-like genes, we have conducted a preliminary study on the expression patterns of members of this gene subfamily in adult mice using specific primer sets and reverse-transcription and polymerase chain reaction technique (Table 2). The expression patterns for *Tspy*, *Tspx*, *Tspy-L1* and *L4* are relatively ubiquitous in many tissues while *Tspy-L3* and *L5* show some tissue-specificity. The detection of transcripts from the *Tspy* gene is somewhat different in other species, including humans, in which the expression of this Y-located gene is restricted to the testis (Zhang et al., 1992; Schnieders et al., 1996; Vogel et al., 1997). Previous studies suggested that the mouse *Tspy* is nonfunctional due to presence of stop codons in its open reading frame (Mazeyrat and Mitchell, 1998; Vogel et al., 1998a). If *Tspy* serves an important role(s) in the physiology of the testis, one of the *Tspy*-like genes could potentially substitute for its function in the mouse testis. Analysis of developmental testes at various ages after birth shows that the expression patterns of both *Tspy* and *Tspy-L5* seem to increase with ages while those for Tspy-L1 and L3 decrease in the same intervals (Figure 5). Both Tspx and *Tspy-L4* show ubiquitous expression patterns throughout these developmental stages. The expression of *Tspy*-like genes in the testis suggests that they could potentially play some roles in the physiology of this organ.

Table 2. Expression of *Tspy and Tspy-L genes* in adult mouse tissues

	brain	thymus	heart	liver	spleen	seminal vesicle	prostate	testis	ovary
Tspx/Tspy-L2	+	+	+	+	+	+	-	+	+
*Tspy**	+	+	+	+	+	-	-	+	-
Tspy-L1	+	+	+	+	+	+	-	+	+
Tspy-L3	+	+	-	-	+	-	-	+	+
Tspy-L4	+	+	+	+	+	+	-	+	+
Tspy-L5	+	-	+	-	-	-	-	+	+

* Pseudogene with in-frame stop codons. +, detectable; -, undetectable by RT-PCR

Despite their differential expression, their exact functions will need to be defined by other experimental means, such disease linkage analysis, as in SIDDT syndrome, and gene knockout studies.

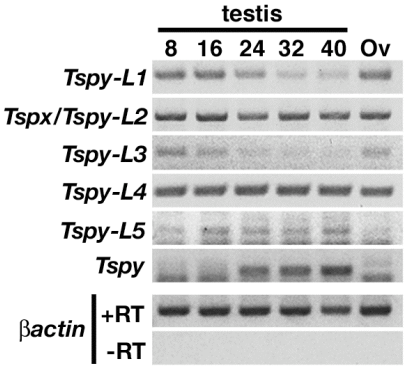

Figure 5. Expression of Tspy and Tspy-like gene in mouse developing testes and adult ovary. RT-PCR analysis with specific primers for respective members of the *Tspy* gene family were performed with total RNAs purified from testes of postnatal mice at 8, 16, 24, 32 days after birth and adult ovaries. β-actin was used as a reference.

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The human Y-encoded testis-specific protein interacts functionally with the putative oncoprotein eEF1A

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Running title: TSPY interaction with eEF1A

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Abstract

Testis-specific protein Y-encoded (TSPY) is the putative gene for the gonadoblastoma locus on the Y chromosome (GBY). TSPY is expressed in normal germ cells of fetal and adult testis and ectopically in tumor germ cells, including gonadoblastoma in intersex patients, testicular germ cell tumors, prostate cancer, and other somatic cancers. As in other members of the TSPY/SET/NAP1 superfamily, it harbors the highly conserved SET/NAP domain. To explore its possible functions in tumorigenesis, we performed a yeast two-hybrid screen of a fetal gonadal cDNA library and identified the translation elongation factor eEF1A as a binding partner for TSPY at its SET/NAP domain. TSPY and eEF1A colocalized in the cytoplasm of and were coimmunoprecipitated from transfected COS7 cells. Immunostaining studies demonstrated that TSPY and eEF1A were highly expressed and colocalized in tumor germ cells of human seminoma specimens, suggesting their possible interaction in germ cell tumors. Significantly, overexpression of eEF1A stimulated the expression of a reporter gene in HEK293 cells, which was exacerbated in the presence of TSPY. As human *EEF1A1* and *EEF1A2* genes are possible oncogenes in various cancers, our results suggest that TSPY may have significant effects on protein synthesis and, by interacting with these putative oncoproteins, may exacerbate eEF1A-associated tumorigenesis.

Introduction

The human testis-specific protein Y-encoded (TSPY) gene was initially identified as a testis-specific gene repeated in tandem on the short arm of the human Y chromosome (1, 2). TSPY is normally expressed in fetal primordial germ cells/gonocytes and adult spermatogonia and spermatids. It has been postulated to perform vital functions in male germ cell development and spermatogenesis (1, 3-5). The TSPY gene cluster has been mapped to the critical region harboring the gonadoblastoma locus on the human Y chromosome (GBY), which harbors genes predisposing the dysgenetic gonads of sex-reversed female and intersex patients to develop gonadoblastoma (6-8). Various studies suggest that TSPY is the putative gene for GBY. TSPY is expressed abundantly in gonadoblastoma, and numerous types of testicular germ cell tumors, including the premalignant precursor, carcinoma *in situ*/intratubular germ cell neoplasia undifferentiated (CIS/ITGCNU), seminomas, and extragonadal intracranial germ cell tumors of male origin (9-11). Its expression is tightly associated with those of key germ cell tumor markers, such as c-KIT and OCT3/4 (9-11). TSPY is also highly expressed in various somatic cancers, including prostate cancer, hepatocellular carcinoma, and melanoma (5, 12-16). Interestingly, transgenic mice harboring a modular transgene directed by a TSPY promoter expressed their transgene in germ cell lineages of both sexes (3), suggesting that ectopic expression of TSPY may contribute to tumorigenesis of gonadoblastoma in an ovarian/dysgenetic gonadal environment. Overexpression of TSPY in cultured cells accelerated cell proliferation and tumorigenicity in athymic mice (17). Currently, the exact mechanisms of its postulated oncogenic or tumor-promoting properties are still unclear.

TSPY shares significant homology with the leukemia patient SE translocation (SET) or template activating factor 1β (TAF-Iβ) oncoprotein and is a founding member of the

TSPY/SET/NAP-1 family (18-20). Members of this gene family encode proteins that harbor a highly conserved SET/NAP domain, present in SET/TAF-Iβ (hereafter referred to as SET) and the nucleosome assembly protein (NAP), and have been demonstrated to perform various cellular functions in regulating gene expression, cell cycle progression, and/or differentiation (17, 18, 21-23). In particular, proteins of the SET/NAP domain have been demonstrated to be histone chaperones and could interact with various transcription cofactors in mediating their respective functions. SET is a subunit of the inhibitor of histone acetyltransferases (INHAT) complex that masks histones from acetylation (23). SET regulates transcription by interacting with transcription factors and coregulators, such as COUP-TF, SF-1, and CREB-binding protein (24, 25). Another member, CINAP (also known as DENTT, TSPY-like2, and TSPX) interacts with CASK, which is a coactivator of the transcription factor Tbr-1 (26). Hence, TSPY/SET/NAP-1 family members could act as scaffolds for transcription regulators (18). Although TSPY shares significant homology at the SET/NAP domain with members of this protein family, it lacks the loosely conserved acidic C-terminal domain that is present among other members (18). Further, SET and CINAP/ TSPX are preferentially located in the nucleus; TSPY is located in both the nucleus and cytoplasm (3, 10, 17). Therefore, TSPY may perform additional functions in the cytoplasm apart from the nuclear functions prescribed for SET and CINAP/TSPX.

To gain insights into possible functions of TSPY, we have performed an interactive cloning study to identify its binding partners using the yeast two-hybrid strategy. Eukaryotic elongation factor 1 alpha (eEF1A) has been identified consistently as an interactive partner for TSPY and is an essential component for the elongation phase during protein translation. Its protein sequence is highly conserved among eukaryotic species ranging from yeast to human. It

binds directly to aminoacyl tRNA (aa-tRNA) in a GTP-dependent manner and brings the aa-tRNA to the A site of the ribosome (27). There are two eEF1A isoforms encoded by two individual genes, *EEF1A1* and *EEF1A2*, located on human chromosome 6q and chromosome 20q, respectively. These isoforms share more than 90% sequence identity at the protein level and could serve essentially the same or similar functions in protein synthesis (28). Importantly, both *EEF1A* genes have been postulated as oncogenes involved in various types of cancer (27). *EEF1A2* is amplified in 25% of primary ovarian tumors and is expressed abundantly in breast cancer and lung adenocarcinoma, suggesting that it could play an important role in oncogenesis in these types of cancers (29-32). NIH3T3 cells overexpressing the eEF1A2 gene are tumorigenic in nude mice, thereby confirming its potential oncogenic roles in human cancers (33). As eEF1A1 and eEF1A2 are highly conserved, eEF1A1 could possess oncogenic functions similar to those of eEF1A2. Indeed, mouse eEF1A1 has been identified as a factor capable of increasing the susceptibility of transformation in mouse fibroblasts by chemical and physical induction (27, 34).

Our results showed that TSPY and eEF1A were colocalized in the same subcellular compartments and coimmunoprecipitated from transfected mammalian cells. Immunostaining analysis showed that TSPY and eEF1A were coexpressed and colocalized in the same tumor germ cells in human seminoma samples. TSPY enhanced the expression of a reporter in HEK293 cells in a dose-dependent manner. These observations suggest that TSPY may exacerbate oncogenic events associated with eEF1A oncogenic actions, thereby promoting tumorigenesis.

Materials and Methods

Gene constructs. The cDNAs encoding for TSPY[full-length], TSPY[residues 76-150], TSPY[residues 151-308], TSPY[residues 151-229], TSPY[residues 190-269], TSPY[residues 230-308], TSPY-like1[full-length], rat Tspy[residues 185-334], CINAP[residues 198-338], and SET[residues 74-223] were generated by polymerase chain reaction (PCR) with specific primers using appropriate full-length cDNA plasmids as templates (Supplemental Table 1). The cDNAs encoding eEF1A1[full-length], eEF1A2[full-length], and eEF1A2[residues 305-464] were generated by reverse-transcript PCR (RT-PCR, Super-Script II , Invitrogen, Carlsbad, CA) or PCR using mouse brain mRNAs. The respective cDNA subclones were ligated into the pGEM-T easy cloning vector (Promega, Madison, WI), and were verified by DNA sequencing.

The pGBKT7 and pGADT7 yeast expression vectors were purchased from Clontech Laboratory (Mountain View, CA). pGBKT7-TSPY[151-308], pGBKT7-TSPY-like1[full-length], pGBKT7-CINAP[198-338], pGBKT7-SET[74-223], and pGBKT7-rat Tspy[185-334] were constructed by inserting the respective cDNAs in-frame into the GAL4 DNA-binding domain. The fetal gonadal cDNA library was constructed in the yeast expression vector, pGADT7, with RNAs (gonad-mesonephros complexes) derived from E11.5 mouse embryos as described previously (35). For glutathione S-transferase (GST)-fusion protein expression, cDNAs encoding TSPY[full-length], TSPY[151-229], TSPY[190-269], TSPY[230-308], CINAP[198-338], or SET[74-223] were ligated into pGEX-4T3 vector (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) or pET41a(+) vector (Novagen, Madison, WI).

The N-terminal FLAG epitope-tagged eEF1A1 (pFLAG-eEF1A1) was constructed by inserting the eEF1A1 cDNA into the p3XFLAG-CMV-7 vector (Sigma-Aldrich, St. Louis, MO).

The C-terminal V5 epitope-tagged eEF1A2 variants (pcDNA-eEF1A2[full-length]-V5 and pcDNA-eEF1A2[305-463]-V5) were constructed by subcloning the appropriate eEF1A2 cDNAs with the pcDNA3.1/V5-His vector (Invitrogen). The TSPY cDNA was inserted into the *pCS2-plus* vector (36, 37), designated as *pCS-TSPY*, for TSPY expression in mammalian cells. The N-terminal FLAG epitope-tagged TSPY[76-150] (*pFLAG-TSPY[76-150]*) was constructed by inserting in-frame the TSPY[76-150] cDNA into the *p3XFLAG-CMV-7* vector. For the human eEF1A1 expression vector *pCS-hEEF1A1*, the human eEF1A1 cDNA was excised from I.M.A.G.E. clone (number 5585079; ATCC, Manassas, VA) using *Not*I and *Sal*I and inserted into the *pCS2-plus* vector, as above.

Yeast two-hybrid screening. A human TSPY cDNA fragment encoding the SET/NAP domain (residues 151-308) was subcloned in-frame into pGBKT7 (pGBKT7-TSPY[151-308]) and was expressed as a fusion protein with the GAL4 DNA-binding domain. Screening of the mouse embryo gonadal cDNA library was performed as previously described (35). Briefly, the bait construct pGBKT7-TSPY[151-308] was transformed into yeast strain Y187 containing mating-type α capable of mating with library strain AH109. After mating, yeast cells were plated on QD selective media (synthetic drop-out media, ΔLeu, Trp, Ade, His) on which yeast cells harboring specific cDNA coding for an interactive protein with TSPY[151-308] would survive. Selected colonies were further tested for α-galactosidase activity on QD/α-galactosidase plates. Colonies showing strong α-galactosidase activity (blue) were further analyzed.

Inserts from selected positive colonies were amplified and propagated in a bacterial host (*Escherichia coli* DH5α, Invitrogen). They were retransformed into AH109 with either pGBKT7-TSPY[151-308] or pGBKT7 vector and plated on QD/α-galactosidase plates to

confirm the interaction among these molecules. The interactions with other TSPY/SET/NAP1 family proteins were similarly tested using pGBKT7-TSPY-like1[full-length], pGBKT7-CINAP[198-338], pGBKT7-SET[74-223], and pGBKT7-rat Tspy[185-334].

GST-pulldown assay. GST-TSPY[full-length], GST-TSPY[151-229], GST-TSPY[190-269], GST-TSPY[230-308], GST-CINAP[198-338], and GST-SET[74-223] were expressed in bacterial host BL21(DE3)pLysS (Novagen) by induction with 1 mM isopropyl-L-thio-β-D-galactopyranoside (IPTG), and were purified with glutathione Sepharose 4B beads (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. The expression vectors for the C-terminal region of eEF1A isoforms, pGADT7-eEF1A1[305-462] and pcDNA-eEF1A2[305-463]-V5, were synthesized by in vitro transcription and translation system (TNT T7 Quick Coupled Transcription/Translation System, Promega) in the presence of 1 μCi/25 μl of ^{35}S-Met according to the manufacturer's instructions. Approximately 10 μg glutathione Sepharose-linked GST or GST-fusion protein was incubated with the ^{35}S-labeled TNT-product (10 μl of reacted solution) in 200 μl of GST binding buffer (PBS, 0.1% NP-40, 5 mM dithiothreitol and protease inhibitors cocktail [Roche, Indianapolis, IN]) with rotation overnight at 4^{o}C. The beads were then washed for 1 hour at 4^{o}C in 500 μL of GST binding buffer. Bound proteins were analyzed by SDS-PAGE and detected with autoradiography.

Coimmunoprecipitation. COS7 cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (HyClone, Logan, UT). The TSPY and FLAG-tagged eEF1A1 expression vectors, pCS2-TSPY (3 μg) and/or pFLAG-eEF1A1 (1 μg),

were cotransfected into COS7 cells using FuGENE6 (Roche). Appropriate empty vectors were used as control. The cells were harvested 48 hours after transfection, washed with PBS, and lysed with the 2.4 ml coIP-buffer (20 mM Tris-HCl [pH 6.5], 300 mM NaCl, 20% glycerol, 1% NP-40) supplemented with a protease inhibitors cocktail (cOmplete Mini, Roche). Lysates (750 μL) were precipitated with 25 μL agarose conjugated with anti-FLAG mouse immunoglobulin (Ig)G (M2, Sigma-Aldrich) at 4^{o}C overnight. The immunoprecipitate was washed with coIP-buffer for 1 hour at 4^{o}C and subjected to SDS-PAGE. Western blot analysis was performed as described previously (38) using anti-FLAG rabbit IgG (1:600, Sigma-Aldrich) and anti-TSPY rabbit serum (1:600) raised against full-length recombinant TSPY protein. Coimmunoprecipitation for FLAG-tagged TSPY[76-150] and full-length TSPY was performed similarly to the method described above, using *p3XFLAG-TSPY[76-150]* and *pCS-TSPY*.

Immunohistochemistry. COS7 cells were seeded 24 hours before transfection on four-well chamber slide (Nalge Nunc International, Rochester, NY), and the epitope-tagged eEF1A expression vectors, pFLAG-eEF1A1 (0. 25 μg) or pcDNA-eEF1A2-V5 (0.25 μg), were co-transfected with pCS-TSPY using FuGENE6. Two days after the transfection, the cells were fixed by 3.7% formaldehyde in PBS for 5 minutes, and then permeabilized by 100% methanol for 5 minutes. After wash by PBS, chamber slides were blocked for 1 hour in 3% bovine serum albumin (BSA) in PBS, and then processed for immunofluorescence with anti-TSPY rabbit serum (1:400), anti-FLAG mouse IgG (1:400, Sigma-Aldrich), and anti-V5 mouse IgG (1:200, Invitrogen). The immunoreactive signals were detected by Alexa Fluor 586 (red)-conjugated anti-mouse IgG and Alexa Fluor 488 (green)-conjugated anti-rabbit IgG (Molecular Probes/Invitrogen, Carlsbad, CA). Fluorescence was examined with an Axiophoto fluorescence

microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) and recorded by a LEI-750 digital imaging system (Leica Microsystems Inc., Bannockburn, IL).

Human testis and seminoma samples were obtained from archival specimens from the Anatomic Pathology Section, VA Medical Center, San Francisco (11). Immunofluorescence of tissue sections was performed and analyzed as described previously (38). For the immunohistological analysis of TSPY, anti-TSPY mouse monoclonal antibody (clone 7) (3) was used. The immunoreactive sites were detected with the SuperPicTure Polymer Detection kit (ZYMED/Invitrogen, Carlsbad, CA) and 3,3'-Diaminobenzidine (DAB) as chromogene, resulting in brown staining. Sections were counterstained with hematoxylin to visualize nuclei (Fisher Scientific, Hampton, NH). The human study was performed under an exempt protocol approved by the Institutional Committee on Human Research, VA Medical Center, San Francisco.

Luciferase assays. HEK293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (HyClone). Cells were seeded at 4×10^4 cells per well on 24-well plate (Nalge Nunc International). The cells were transfected with 5 ng/well of *pRL-TK* (Promega) and various amounts of *pCS-TSPY* and/or *pCS-hEEF1A1* plasmids using siPORT XP-1 transfection reagent (Ambion, Austin, TX). Forty-eight hours later, cell were washed with 500 µl PBS, harvested in 100 µl of reporter lysis buffer (Promega), and stored as complete cell lysis at –80°C. The luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega) and TD-20/20 luminometer (Turner Biosystems, Sunnyvale, CA) according to the manufacturers' instructions.

Results

Identification of the eukaryotic eEF1A1 as an interactive partner for TSPY. To elucidate the molecular mechanisms responsible for TSPY functions, we have performed a yeast two-hybrid screen of a mouse fetal gonadal cDNA library using the TSPY SET/NAP domain as bait. Forty clones of 130 well-grown blue colonies were randomly selected for DNA sequencing. One of these clones encodes the C-terminus region (residues 305-462) of eEF1A1. Further analysis of all 130 colonies by PCR showed that four of 130 harbored eEF1A1 cDNA. AH109 yeast cells transformed with either pGBKT7-TSPY[151-308] or pGADT7 full-length eEF1A1 showed negative growth on selection medium (data not shown). eEF1A1 is one of two isoforms (eEF1A1 and eEF1A2) of eEF1A. eEF1A1 is ubiquitously expressed, while eEF1A2 is differentially expressed, primarily in the postnatal stages (39). eEF1As are mostly localized in the cytoplasm and are important in the recruitment of aa-tRNA in protein synthesis and nuclear export (40, 41).

As the SET/NAP domain of TSPY shares significant homology with those of other proteins of the TSPY/SET/NAP1 family (Fig. 1*A*), we further explored possible interactions between eEF1A1[305-462] and other TSPY/SET/NAP1 family members, such as TSPY-like1, CINAP/TSPX, SET and rat Tspy using the yeast two-hybrid strategy. Our results showed that only the human TSPY[151-308] and rat Tspy[185-334] interacted with eEF1A1[305-462] in yeast (Fig. 1*B*).

***In vitro* mapping of the interactive domains between TSPY and eEF1As.** *In vitro* GST pulldown assays were used to confirm the interaction between TSPY and eEF1A and to delineate the respective interactive domains. An interaction was observed between eEF1A1[305-462] and

GST-TSPY[full-length], but not with GST alone (Fig. 2*B*). To map the interactive domains of TSPY responsible for its eEF1A1 binding, a series of GST-TSPY truncated mutants, TSPY[151-229], GST-TSPY[190-269] and GST-TSPY[230-308], were generated and used as bait in respective pulldown assays with ^{35}S-labeled eEF1A1[305-462]. Both GST-TSPY[190-229] and GST-TSPY[230-308], which contain highly conserved sequences with other TSPY/SET/NAP1 family (Fig. 1*A*), could bind eEF1A1[305-462] effectively (Fig. 2*C*, lanes 3 and 5 in top). TSPY mutants lacking either region [151-189] or [270-308] showed no or faint binding to eEF1A1[305-462] (Fig. 2*C*, lane 4 at top).

The analogues eEF1A1 and eEF1A2 share >90% identity and have essentially the same function in protein translation (28). To explore the possibility of an interaction between TSPY and eEF1A2, the eEF1A2[305-463] expression vector pcDNA-eEF1A2[305-463]-V5 was generated, translated *in vitro* with ^{35}S-methionine, and used similarly in the GST pulldown assays with a series of GST-TSPY mutants, as described above. The results showed that eEF1A2[305-463] is capable of binding to TSPY at domains as specific as those for the eEF1A1 (Fig. 2*C*, lanes 3 and 5 at bottom), as summarized in Fig. 2*D*.

Although eEF1A1 did not show significant *in vivo* binding to the conserved SET/NAP domains of other members of the TSPY/SET/NAP family, we explored the possibility that they might interact *in vitro* in GST pulldown assays. Our results showed that GST-SET[74-223] (Fig. 2*C*, lane 7) and GST-CINAP[198-338] (Fig. 1*C*, lane 6) bound readily with eEF1A1[305-462]. Our observation is consistent with those described in a recent report on SET-binding proteins analyzed by proteomics strategies (42). Currently, we are uncertain of these differences between *in vivo* yeast two-hybrid and *in vitro* GST pulldown approaches on the interactions between eEF1A1 and SET/CINAP. Interestingly, both CINAP/TSPX and SET are localized in the nuclei,

whereas eEF1A is restricted to the cytoplasm. Further cellular and physiological analyses will be required to elucidate the significance, if any, of the interaction among these molecules.

TSPY colocalizates and interacts with eEF1A and forms homodimers in mammalian cells. To confirm the interaction between TSPY and eEF1A in mammalian cells, immunofluorescence was performed on COS7 cells transfected with TSPY and FLAG-eEF1A1 or eEF1A2-V5 expression vectors. eEF1As exhibited mostly cytoplasmic staining (Fig. 3*A* and 3*B*, FLAG-eEF1A1 or eEF1A2-V5, respectively) while TSPY was localized in both the nucleus and cytoplasm. The cytoplasmic TSPY exhibited an almost identical staining pattern with both eEF1As (Fig. 3*A* and 3*B*, TSPY), as revealed on merged images of TSPY and eEF1As (Fig. 3*A* and 3*B*, yellow dots in the merged images). To confirm their interactions in the transfected cells, total cell lysates were coimmunoprecipitated with an anti-FLAG antibody. The precipitates were then analyzed by Western blot using anti-TSPY antibody. The result showed that TSPY was pulled down with FLAG-eEF1A1, but not in the absence of FLAG-eEF1A1 (Fig. 3*C*), thereby confirming an interaction between TSPY and eEF1A in transfected COS7 cells.

Recently, the atomic structure of the functional domain of the human SET protein was elucidated (43). The SET protein forms homodimers through their α2-helices located on the N-terminal region (43). Using the web-based Protein Homology/analogy Recognition Engine (Phyre) program (http://www.sbg.bio.ic.ac.uk/phyre/html/index.html) (44), we analyzed the TSPY structure using the SET atomic structure as a model. Our analysis identified two long α-helices (α1 and α2) at residues 52-95 and 105-141 of TSPY, analogous to those of SET (Supplemental Figure S1). To explore the possibility that TSPY could indeed form homodimers, the interaction between the full-length TSPY and an abbreviated form, FLAG-

TSPY[76-150], was examined by coimmunoprecipitation, similarly to the method described above. Our results showed that the full-length TSPY was pulled down with FLAG-TSPY[76-150] (Fig. 3*D*), suggesting that TSPY could form homodimers through its N-terminal domain.

TSPY and eEF1A are coexpressed in testicular germ cells and germ cell tumors. TSPY is the putative gene for GBY and is abundantly expressed in both premalignant precursors and tumor cells in gonadoblastoma and seminomas. As both EEF1A1 and EEF1A2 are postulated to be oncogenes (27, 29, 30, 32, 33), we have explored the possibility that they are also expressed in germ cell tumors using immunological approaches and anti-TSPY and anti-eEF1A antibodies. In normal testis, TSPY was detected in a germ cell–specific manner, as we reported previously (Fig. 4*A* and 4*B*, TSPY) (3). eEF1A was preferentially expressed in germ cells, as compared to the surrounding peritubular myoid cells (Fig. 4*A* and 4*B*, eEF1A). TSPY was distributed in both the nuclei and cytoplasm of germ cells, while eEF1A was detected in the cytoplasm, which is similar to their respective distribution in transfected COS7 cells. Under high magnification, TSPY and eEF1A exhibited colocalization in the cytoplasm (Fig. 4*B*, merged image).

As discussed above, TSPY is highly expressed in testicular germ cell tumors (e.g. seminoma) and their premalignant precursors (i.e. CIS/ITGCNU). To explore the possibility that eEF1A is also expressed at elevated levels in testicular germ cell tumors, five cases (SE#1-5) of seminoma and three cases of nonseminoma samples were analyzed by immunofluorescence using anti-TSPY and anti-eEF1A antibodies. In all cases, eEF1A and TSPY were consistently expressed in the same tumor cells in both seminoma and nonseminoma samples. TSPY was specifically expressed in the tumor cells of both CIS/ITGCNU and seminoma in the seminomatous specimens, and those of CIS/ITGCNU in nonseminomas, as reported previously

(Fig. 5*A*, TSPY) (11). Similarly, eEF1A was highly expressed in seminoma and CIS/ITGCNU areas (Fig. 5*A*, eEF1A), corresponding to those positive for TSPY (Fig. 5*B*), in addition to a general low-level expression elsewhere within these testicular germ cell tumors. Under high magnification, we observed that much of the cytoplasmic immunostaining signals of both proteins were colocalized, as revealed on the merged image (Fig. 5*C*). Interestingly, the premalignant precursor, CIS/ITGCNU, showed almost identical staining patterns for both eEF1A and TSPY, compared with surrounding TSPY-negative cells (Fig. 5*D*, arrowheads, and Supplemental Figure S2). These results suggest that TSPY and eEF1A are expressed and colocalized in the same tumor cells in testicular seminomas.

TSPY stimulated synthesis of reporter protein in HEK293 cells. Because eEF1A is an important component of the cellular translational machinery, by interacting with eEF1A, TSPY could affect the overall protein synthesis in the cell. To examine this possibility, we characterized the effect of ectopic TSPY expression on protein synthesis, in terms of the levels of a luciferase reporter activity in cultured cells. HEK293 cells were transiently cotransfected with either the pCS-TSPY or pCS-hEEF1A1 construct, a combination thereof (0–0.4 μg/well) and pRL-TK (5 ng/well) in quadruplicate. The inclusion of the pRL-TK plasmid was designed to provide low to moderate levels of luciferase expression under the control of Herpes simplex virus thymidine kinase promoter. Forty-eight hours after transfection, the cells were lysed and analyzed for luciferase activity. When pRL-TK was cotransfected with the human eEF1A1 expression vector pCS-hEEF1A1, the transfected cells showed greater luciferase activity (Fig. 6, columns 6 and 7) than did the control (Fig. 6, column 1), suggesting that increased expression of eEF1A enhanced the protein synthesis in HEK293 cells. To evaluate the effect of TSPY on

protein synthesis, pCS-TSPY was cotransfected with the luciferase reporter. Our results demonstrated that ectopic expression of TSPY stimulated luciferase activities in a dose-dependent manner (Fig. 6, columns 1–5) that was presumably proportional to the indirect effects of TSPY on the synthesis of this reporter. Significantly, TSPY could further enhance the stimulation of eEF1A1 on the expression of luciferase in the transfected cells (Fig. 6, columns 8 and 9). These observations suggest that TSPY could enhance protein production via its interaction with eEF1As, components of the protein translational machinery in the cell.

Discussion

The TSPY/SET/NAP1 family members harbor a conserved SET/NAP domain but serve a variety of diverse cellular functions, including transcriptional regulation, cell cycle regulation, and chromatin assembly/remodeling (17, 18, 21-23, 25). Currently, the exact mechanisms by which they mediate their biological functions are unknown. Selected members, such as SET and CINAP/TSPX, are localized in the nucleus and serve as histone chaperones involved in histone storage and chromatin assembly/remodeling (18, 21, 23, 25), and as coregulators of steroid hormone receptor functions, and modulation of gene transcription (45, 46). The Y-encoded TSPY, on the other hand, is distributed to both the nucleus and cytoplasm, suggesting that it could serve multiple functions in both cellular compartments. Indeed, TSPY has been demonstrated to bind core histones (38), and hence could potentially serve as a histone chaperone in the above mentioned functions, as do the other members of this protein family. Its cytoplasmic location, however, suggests that it could play additional and undefined roles in other cellular functions.

In the present study, we have identified eEF1A as a binding partner for TSPY. eEF1A1 and the highly homologous eEF1A2 are important components of the translational machinery and postulated oncogenes in various human cancers. Our results clearly demonstrate that TSPY interacts with eEF1As and affects their protein synthetic functions. The elucidation of the atomic structure of human SET functional domain (43) has greatly enhanced the opportunity to explore the structure-function relation between the interactions of TSPY and eEF1A. The SET homodimer forms a headphone-like structure consisting of the N-terminus, a backbone helix, and an earmuff domain (Supplemental Figure S1) (43). The SET/NAP domain constitutes the earmuff structure, critical for its histone chaperone activity (18, 43). SET and NAP1 form homodimers through their respective helix-α2s localized on their N-terminal regions (18, 43). The present study shows that TSPY is capable of forming homodimers at its N-terminal region and that it binds eEF1A at domains corresponding to helix-α3, -α 4, -α 5, -α 7 and -α8 of SET, localized at the bottom of the earmuff structure (Supplemental Figure S1) (43). Therefore, it is conceivable that TSPY could form a headphone-like homodimer and bind two eEF1A molecules at its SET/NAP domains. Currently, the physiological effects of such binding are uncertain.

EEF1A genes encode essential factors in translation elongation and are potential oncogenes. Abnormally high expression of eEF1A is frequently observed in various human cancers (i.e., ovarian cancer, breast cancer, and lung adenocarcinoma) (30-33). The eEF1A proteins are critical for cell survival. In mouse neurons, there is a switch from eEF1A1 to eEF1A2 at the early postnatal stage (39). Deletion of the eEF1A2 promoter results in the wasted mouse (*wst*/*wst*), which suffers from immunodeficiency and neurological abnormalities at early postnatal stages and which usually does not survive beyond 30 days of age (47). Various studies suggest that eEF1A activities are closely associated with cell proliferation and cell survival (48).

Cell proliferation requires adequate protein synthesis to fulfill metabolic and growth requirements (49). Other factors within the protein synthetic machinery, such as translation initiation factor eIF4E, are also expressed at high levels in numerous cancer types, thereby promoting tumorigenesis through enhancement of the synthesis of essential growth factors (50). The present study shows that eEF1A is also highly expressed in both premalignant precursor (CIS/ITGCNU) and tumor cells of human testicular seminoma samples (Fig. 5 and Supplemental Figure S2). Significantly, its expression is closely correlated with that of its interactive partner TSPY, which is capable of modulating its protein synthetic functions (Fig. 6), suggesting that eEF1As potentially may play a role in germ cell tumorigenesis.

The eEF1A proteins play an important role in the channeling of aa-tRNA during the protein elongation phase. Following the GDP-to-GTP exchange by eEF1B, eEF1A receives an aa-tRNA directly or indirectly from aa-tRNA synthetase, and recruit it to the ribosomal A-site (27, 41, 51). It was suggested that eEF1B binds on the domain-II of eEF1A prior to the recognition site of domain-I, and sequentially an aa-tRNA zips in the eEF1B-binding pocket of domain-II (51). Since the TSPY binding region of eEF1A locates within [residues 305-462] that is adjacent to the eEF1Bα/aa-tRNA binding region (Fig. 2*A*), it is expected that TSPY may affect on the channeling function of eEF1A by modulating the interactions between eEF1A and its interactive proteins (i.e. eEF1B and ribosomal subunit). In addition to its protein synthetic function, eEF1A could perform other vital cellular functions, including Akt activation and proteasome-mediated protein degradation (52, 53). By interacting with eEF1A, TSPY could have significant effects on these eEF1A-related cellular processes.

Our results show that TSPY and eEF1A is colocalized in the same subcellular compartments and coimmunoprecipitated from transfected mammalian cells. Immunostaining

analysis showed that TSPY and eEF1A were coexpressed and colocalized in the same tumor germ cells in human seminoma samples. TSPY enhanced the expression of a reporter in HEK293 cells in a dose-dependent manner. These observations suggest that TSPY may exacerbate oncogenic events associated with eEF1A oncogenic actions, thereby promoting tumorigenesis. Further studies of the interactions between TSPY and eEF1A could potentially result in significant insights into the role of eEF1A in the functions of TSPY in normal physiology and in tumorigenesis of testicular germ cell tumors, prostate cancer, and other somatic cancers, such as melanoma and hepatocellular carcinoma (12, 13, 16).

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Figure Legends

Figure 1.

The SET/NAP domain of human TSPY and eEF1A1 interact in yeast. *A*, amino acid sequence alignment of SET/NAP domains of human TSPY/SET/NAP1 family proteins and rat Tspy (rTspy). The amino acid residues highlighted in black are conserved, and those regions with conserved chemical properties are shown in grey. The regions of TSPY used in GST pulldown assays are indicated by gray lines. The MacVector Sequence Analysis Program (MacVector Inc.) was used for generation of the sequence alignment. *B*, yeast colonies that grew on plates lacking tryptophan and leucine (ΔTrp, Leu) were examined for growth on adenine- and histidine-deficient plates (ΔAde, His, Trp, Leu). Growth in the absence of leucine indicates the presence of pGADT7 or pGADT7-eEF1A1[305-C], whereas growth in the absence of tryptophan indicates the presence of pGBKT7 containing the indicated derivatives of TSPY/SET/NAP1 family proteins. Interactions between proteins are shown by the ability to activate the *ade2* and *his3* reporter genes. Abbreviations; control, pGBKT7 vector alone; TSPY, pGBKT7-TSPY[151-308]; TSPY-L1, pGBKT7-TSPY-like1[full-length]; CINAP, pGBKT7-CINAP[198-338]; SET, pGBKT7-SET/TAF-Iβ[74-223]; rTspy, pGBKT7–rat Tspy[185-334].

Figure 2.

The C-terminus region of eEF1A interacts with the SET/NAP domains of TSPY and CINAP/TSPX *in vitro*. *A*, domain structure of the full-length eEF1A. The positions of amino acid residues were derived from computer alignment with yeast eEF1A (GenPept accession number; NP_009676) using the MacVector program. The region harbored in the cloned pGADT7-cDNA library is indicated by the bold bar (amino acid residues 305-462 of eEF1A1).

The regions corresponding to the eEF1Bα-, GTP-, and aa-tRNA-binding sites of yeast eEF1A are shaded in gray; [residues 22-167] and [252-322], respectively (referred to Andersen *et al.* (51)). *B*, GST alone or the indicated GST-fusion proteins immobilized on glutathione-agarose beads were incubated with ^{35}S-labeled eEF1A1[305-462]. After pulldown, the bound proteins were resolved by SDS-PAGE and detected by autoradiography. The C-terminus region of eEF1A1[305-462] bound specifically to full-length TSPY. Input lane represented 8% of the peptides used for the pulldown assay. *C*, GST pulldown was performed as in *B* with the indicated GST-fusion proteins and ^{35}S-labeled eEF1A1[305-462] or eEF1A2[305-463]. The TSPY regions used in the binding assays with eEF1A are illustrated as shown at the bottom.

Figure 3.

TSPY colocalized and interacted with eEF1A in mammalian cells. *A*, COS7 cells were transiently cotransfected with TSPY and FLAG-tagged eEF1A1 expression vectors. Cells were examined 2 days later by indirect immunofluorescence for the cellular distribution of TSPY and eEF1A1 using anti-TSPY and anti-FLAG antibodies. *B*, the cellular distribution of TSPY and eEF1A2 in COS7 cells was analyzed as in *A* with anti-TSPY and anti-V5 antibodies. TSPY and eEF1A were well colocalized in the cytoplasm of transfected cells, resulting in a yellowish color in the merged images. Scale bar= 25 μm. *C*, COS7 cells were cotransfected with the indicated combinations of plasmids (TSPY, pCS2-TSPY; FLAG-eEF1A1, pFLAG-eEF1A1; -, appropriate empty vector). Lysates were collected at 2 days and examined by Western blot for expression of TSPY (input, top) and FLAG-eEF1A1 (input, bottom). Immunoprecipitation was performed with anti-FLAG antibody to detect an interaction between TSPY and eEF1A1. The interacting proteins were analyzed by Western blot using anti-TSPY and anti-FLAG antibodies (pulled

down). TSPY was specifically pulled down with FLAG-tagged eEF1A1 (*lane 5*). *D*, COS7 cells were cotransfected with the indicated combinations of plasmids (TSPY, pCS2-TSPY; FLAG-TSPY[76-150], pFLAG-TSPY[76-150]; -, appropriate empty vector). Interaction between TSPY and TSPY[76-150] was analyzed by immunoprecipitation as described in *C*. Full-length TSPY was significantly co-immunoprecipitated with FLAG-tagged TSPY[76-150] (*lane 5*, arrow). Open arrowhead indicates the IgG bands originated from anti-FLAG antibody used in immunoprecipitation.

Figure 4.

TSPY is coexpressed with eEF1A in normal testicular germ cells. *A*, a representative example of the immunofluorescence of TSPY (green) and eEF1A (red) in normal human testis. The merged image shows the signals of anti-TSPY (green), anti-eEF1A (red), and DAPI for DNA staining (blue) altogether. Peritubular myoid cells (Pm) were stained faintly by anti-eEF1A antibody. *B*, highly magnified images of the boxed area in *A*. TSPY and eEF1A were well colocalized in the cytoplasm of spermatogonia (Sg) and spermatocytes (Sc) (yellowish color in the merged images). St indicates the nucleus of a Sertoli cell. *C*, images of the negative control (without primary antibody). Scale bar= 200 μm in *A* and *C*.

Figure 5.

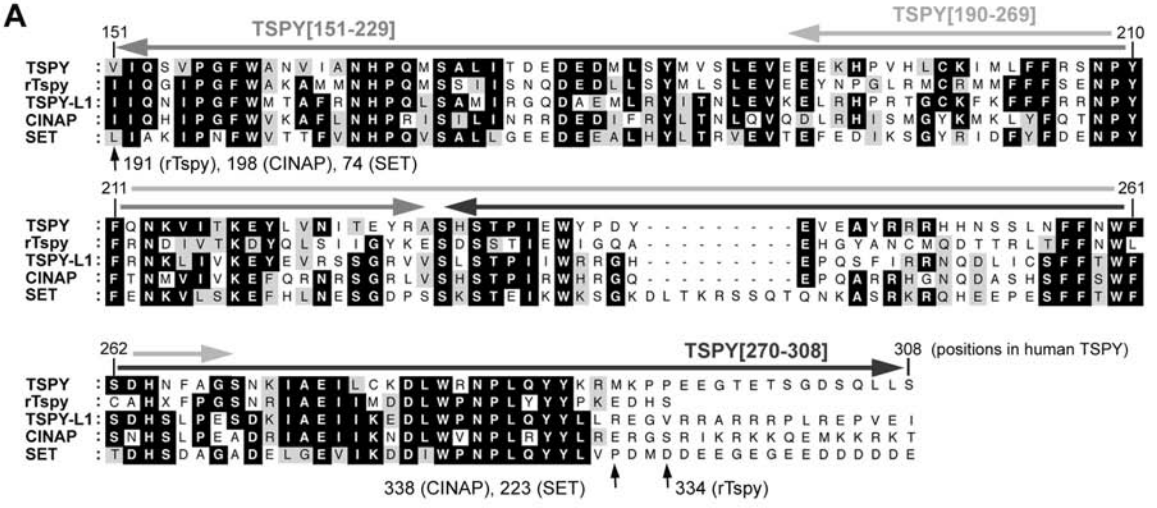
TSPY is coexpressed with eEF1A in human germ cell tumor cells. *A*, representative example of immunohistochemistry of TSPY or eEF1A in human seminoma (Se) and CIS/ITGCNU (CI). Both TSPY and eEF1A were detected in the TSPY-positive cells of the seminoma and CIS/ITGCNU. The control image presents a negative control using nonimmunized mouse IgG

for primary antibody. *B* and *C*, images of immunofluorescence of TSPY (green) and eEF1A (red) in seminoma (neighboring area of box b in panel *A*) at low magnification and high magnification, respectively. Merged images show the signals of anti-TSPY (green), anti-eEF1A (red), and DAPI for DNA staining (blue) altogether. *D*, images of immunofluorescence of TSPY (green) and eEF1A (red) in CIS/ITGCNU (neighboring area of box d in panel *A*). TSPY and eEF1A were well colocalized in the cytoplasm of germ cell tumor cells, resulting in a yellowish color in the merged image. TSPY-positive cells of CIS/ITGCNU are stained stronger by anti-eEF1A antibody than are neighboring TSPY-negative cells (arrowheads). Scale bar= 200 μm in *B* and *D*.

Figure 6.

Stimulation of the model protein synthesis by eEF1A and TSPY. HEK293 cells were cotransfected with pRL-TK (5 ng/well) in the presence of various amounts of pCS-TSPY and/or pCS-hEEF1A1 as indicated in the figure. Total DNA was adjusted with empty vector (pCS2-plus). Luciferase activities were measured 2 days after transfection. The indicated relative activity is based on the luciferase activity of the control (0 μg/well of both pCS-TSPY and pCS-hEEF1A1). The data represent the means ± SE of four individual experiments. Lower panel shows the expression level of TSPY and α-tubulin as an internal control.

Kido T, Figure 1



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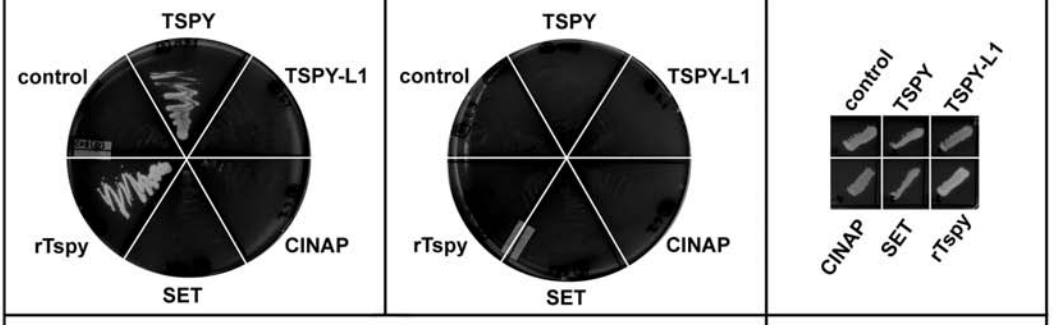
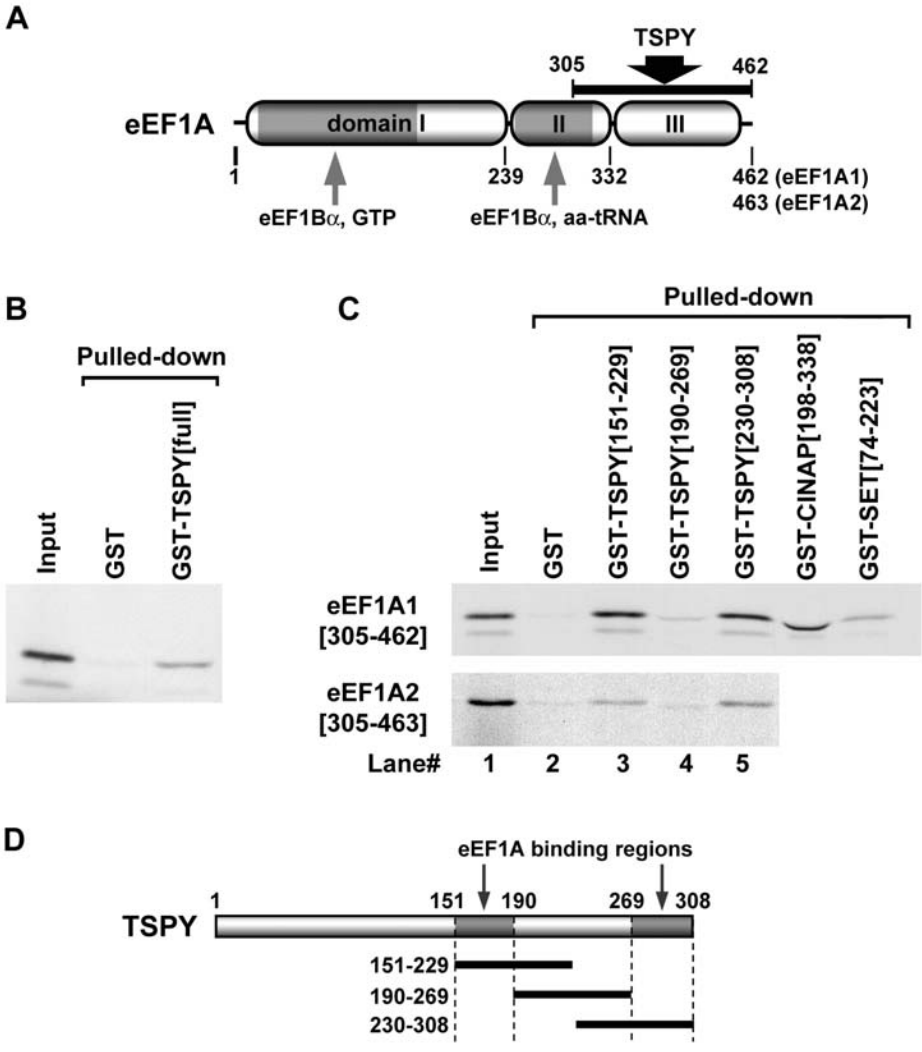


	pGADT7-eEF1A1[305-C]	*pGADT7*
TSPY	+	-
TSPT-L1	-	-
CINAP	-	-
SET	-	-
rTspy	+	-

Kido T, Figure 2



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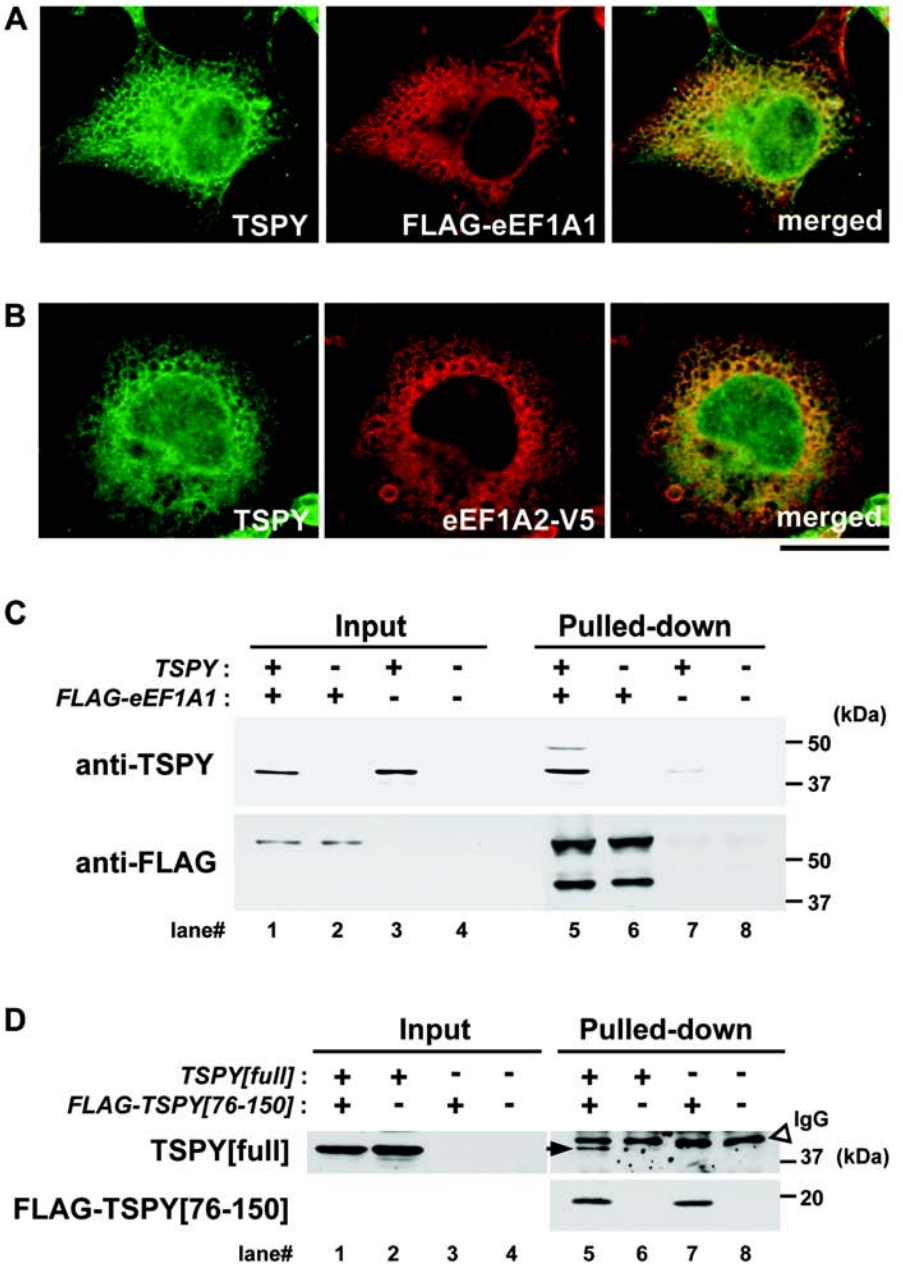

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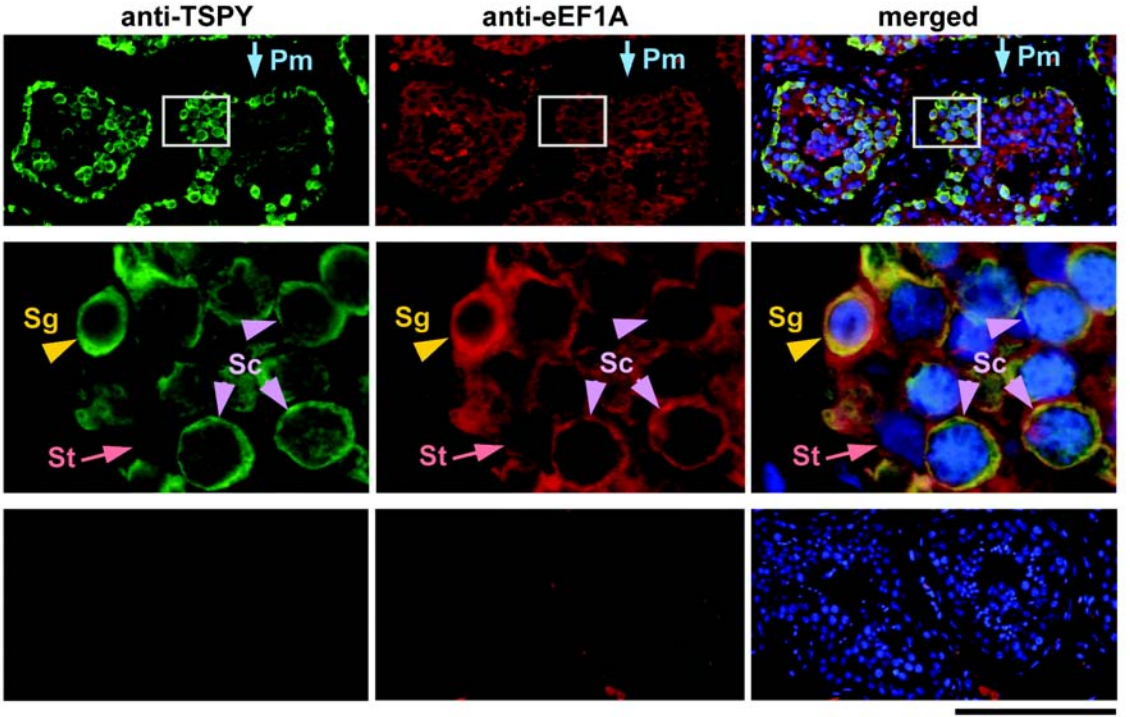

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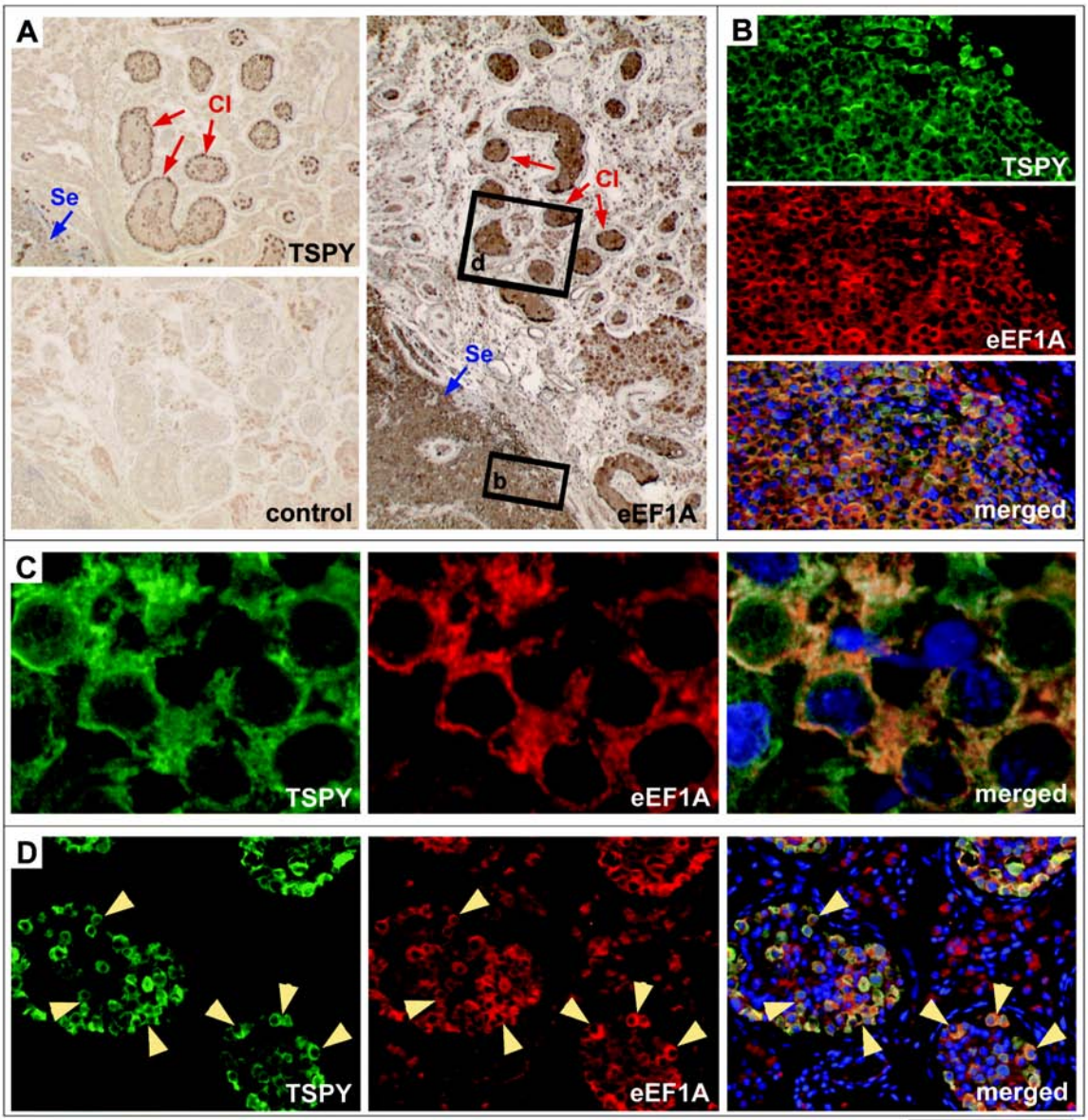

Kido T, Figuer 6